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# Development of probiotics for the sustainable cultivation of pirarucu, *Arapaima gigas*

by

**Gabriella do Vale Pereira**

A thesis submitted to the University of Plymouth in partial fulfilment for the degree of

**DOCTOR OF PHILOSOPHY**

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Development of probiotics for the sustainable cultivation of pirarucu, *Arapaima gigas*.
by Gabriella do Vale Pereira

## Abstract

The pirarucu (*Arapaima gigas*) from Amazon basin is currently the largest farmed fish species and its production is increasing rapidly in Brazil. However, there is a concern about bacterial disease outbreaks and resulting mortalities in pirarucu farms. The use of probiotic bacteria as prophylactic method is recognized as beneficial practice to enhance fish production. The aim of this thesis was to isolate autochthonous probiotic strains from the pirarucu intestine, characterize their potential probiotic characteristics *in vitro*, and perform *in vivo* colonization and growth experiments. To this end, the intestinal microbial community of *A. gigas* was assessed at two different growth stages using high-throughput sequencing (HTS) analysis. Proteobacteria, Fusobacteria and Firmicutes were the most abundant phyla. At genus level *Bradyrhizobium* and *Cetobacterium* were the most abundant in adult and juvenile fish, respectively. In a further trial two isolated lactic acid bacteria (LAB): *Lactococcus lactis* subsp. *lactis* and *Enterococcus faecium*, showed *in vitro* suitability as probiotics. Two potential pathogens: *Citrobacter freundii* and *Pseudomonas* sp., were also isolated and their pathogenicity assessed. Subsequently, an *in vivo* experiment was performed to assess the potencial of LAB strains to colonise and modulate the gut microbiome of pirarucu after 21 days of feeding. The results showed a high abundance of *Cetobacterium* in all treatments. Additionally, both probiotic treatments decreased the levels of Clostridiales in pirarucu intestine and showed adherence to the fish mucosal tissue. Finally, a growth experiment was performed to assess the ability of to the candidate probiotics to improve growth parameters after 42 days of feeding. HTS confirmed that *Cetobacterium* was the most abundant genus in all treatments. Fish fed with *L. lactis* subsp. *lactis* presented higher percentage of increase (%I) of weight, specific growth rate (SGR), and monocytes in blood. The strain *E. faecium* interacted with the microbial gut community and was able to populate the mucosal tissue. In conclusion, both LAB strains presented probiotic characteristics and should be considered as probiotics in *A. gigas* farming. These probiotics could contribute to a reduction in antibiotics use in pirarucu farms, thus, adding value to the species as a sustainable aquaculture product.

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## List of Abbreviations

| | |
|---|---|
| ANOVA | Analysis of variance |
| cDNA | Complementary deoxyribonucleic acid |
| CFU | Colony forming unit |
| EFSA | European Food Safety Authority |
| FAO | Food and Agriculture Organization of the United Nations |
| FCR | Feed conversion ratio |
| GALT | Gut-associated lymphoid tissue |
| GI | Gastro-intestinal |
| HTS | High-throughput sequencing |
| IEL | Intra epithelial leucocyte |
| LAB | Lactic acid bacteria |
| LEfSe | Linear discriminant analysis effect size |
| OTU | Operational taxonomic unit |
| PBS | Phosphate buffered saline |
| PCoA | Principal coordinates analysis |
| PCR | Polymerase chain reaction |
| PD | Phylogenetic diversity |
| QIIME | Quantitative insights into microbial ecology |
| RNA | Ribonucleic acid |
| RT-qPCR | Quantitative real-time polymerase chain reaction |
| SD | Standard deviation |
| SGR | Specific growth rate |
| WG | Weight gain |

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Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.

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**2) SfAM Early Career Scientists (ECS) Meeting 2017.**Autochthonous probiotic bacteria and modulation of intestinal microbiota of Pirarucu (*Arapaima gigas*).

**3) Aquaculture Europe 2016.** Intestinal microbiota of pirarucu *Arapaima gigas* using high-throughput sequencing analysis.

**4) SfAM Early Career Scientists (ECS) Meeting 2016.** (Attendance).

**5) Aquaculture Europe 2015.** Selection of lactic acid bacteria from the intestine of pirarucu, *Arapaima gigas*.

**6) Aquaculture Europe 2014.** Characterization of lactic acid bacteria isolated from the intestine of pirarucu, *Arapaima gigas*.

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The carried out during this thesis were performed in collaboration with Federal University of Santa Catarina (UFSC - Brazil) and the pirarucu cultivation farm *Mar & Terra Ind. Com. de Pescados* Located in Rondonia state, Brazil.

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## Publications

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Rodiles A., Rawling M. D., Peggs D. L., Pereira G. do V., Voller S., Yomla R., Standen B. T., Bowyer P., and Merrifield D. L. 2018. Probiotic Applications for Finfish Aquaculture. In: Probiotics and Prebiotics in Animal Health and Food Safety. 1ed.: Springer International Publishing, 2018, v.1, p. 197-217.

# Chapter 1:
# General Introduction

# Chapter 1: General Introduction

## 1.1 Aquaculture Production in South America

Aquaculture is the activity of cultivating plants and animals whose lifecycle is partly or completely aquatic. According to the Food and Agriculture Organization of the United Nations (FAO), aquaculture is considered one of the fastest growing food industries, currently comprising about 50% of the world's fish production (FAO 2017a).

In South America, in the period 2012 to 2014, the Republic of Chile represented 43.4% of the total volume of Latin American and Caribbean aquaculture alone, followed by the Republic of Brazil (19.9%) and the Republic of Ecuador (13.4%). These three countries dominate aquaculture production in South America accounting almost 77% of the total volume at the same period (FAO 2017a). Within the countries mentioned, three species dominate aquaculture production. These are: shrimp (*Penaeus vannamei*), Atlantic salmon (*Salmo salar*), and Tilapia (*Oreochromis* spp.) representing 55% of the total production (FAO 2017a).

Although Brazil has vast areas and, volumes of freshwater and an extensive coast, Brazilian aquaculture production only began to burgeon in the late 1980's. Total production in Brazil (including fish, crustaceans, molluscs, aquatic plants, etc.) in 2015 reached 574,530 tonnes. Within this total, the production of fresh water fish contributed the majority of volume, accounting for 483,606 tonnes (FAO 2017) (Figure 1.1). Fish production in Brazil accounted for 83% of the total aquaculture output in 2013 (IBGE 2015). Within this total, fresh water fish are predominant in Brazil and exotic fish such as Nile tilapia (*Oreochromis niloticus*) are almost half of the total production (47%).

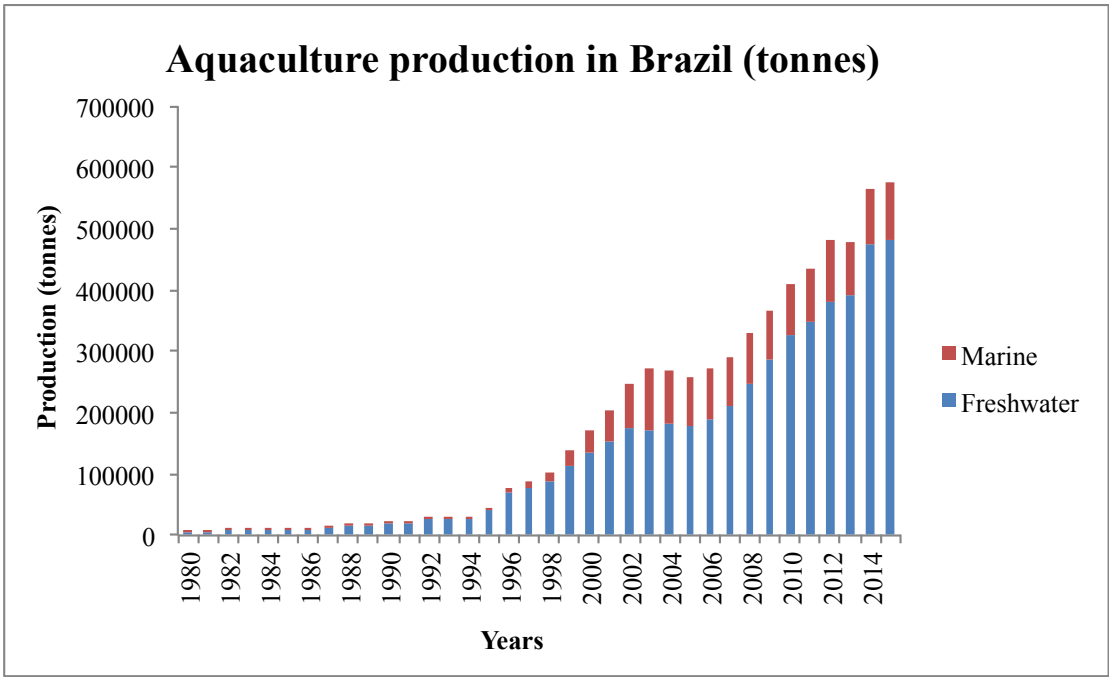



Figure 5.1: Aquaculture production in Brazil (tonnes) from 1980 to 2015 (FAO 2017)

In recent decades Brazilian aquaculture companies have been investing in the production of native fish, mainly for exportation to Europe and United States. Species such as: hybrid surubim (*Pseudoplatystoma corruscans* × *Pseudoplatystoma reticulatum*) (Pereira et al., 2015), pacu (*Piaractus mesopotamicus)* (Portella et al., 2014), jundiá or silver catfish (*Rhamdia quelen*) (Barcellos et al., 2004) and tambaqui (*Colossoma macropomum*) have been studied focusing in their viability in aquaculture (de Oliveira et al., 2012). Amongst the native species that have been considered, the pirarucu fish (*Arapaima gigas*), has great potential.

The giant Amazon native fish, pirarucu (*Arapaima gigas*), appears to have a promising potential due to its fast growth rate (up to 12 kg/year) (Bezerra et al., 2013). According to the FAO, the Brazilian production of pirarucu was 8,387 tonnes in 2015 and yielded USD 25,758 000 dollars at the same year (FAO 2017). The absence of intramuscular bones in the flash of pirarucu also assures a greater market value and increased exportation standards reaching prices from USD 12-15/kg in South American cities and USD 20-25/kg in Europe and the United States of America.

## 1.2 Pirarucu (*Arapaima gigas*)

### Natural history

Pirarucu is a teleost fish beloning to the Arapamidae (Osteoglossidae) and Actinopeterygii Sub-Class (Bezerra et al., 2013). It is phylogeneticaly related with African bonytongue (*Heterotis niloticus*) and Arawana (*Osteoglossum bicirrhosum*) (Broughton et al., 2013).

*A. gigas* (Figure 1.2) is endemic to the Amazon basin, it is one of the largest freshwater fishes in the world, reaching a length of 3 m and weighing as much as 250 kg (Brauner et al. 2004). *A. gigas* is a water-oxigen breather until around nine days post-hatch, and then it becomes an obligate air-breathing fish (Brauner et al. 2004). Due to the presence of capillaries leading to its modified swim bladder, this fish is able to perform gaseous exchange with the surface oxygen allowing the fish to breath, leaving the gills to act as a secondary respiratory organ (Bezerra et al., 2013).


Figure 1.6: *Arapaima gigas.* Tom Dolan/Encyclopædia Britannica, Inc., painted under the supervision of Loren P. Woods, Chicago Natural History Museum*

### Growth performance

One of the first studies available on this species characterised the fish in its natural habitat and emphasized its substantial potential for aquaculture (Saint-Paul 1986). For example, the pirarucu can grow from 19 g to 2560 g in 10 months in a recirculating warm water culture system (Saint-Paul 1986). In 2010, a Brazilian Government agency (SEBRAE 2010) published a handout in which they described the pirarucu growth in different culture systems based on studies during three years in Brazilian farms. The pirarucu growth performance after 12 months either in pond, cages or in recirculation systems is very impressive (Table 1.1). In this context, Burton et al. (2016) reported the *A. gigas* final weight of 6.56 kg (initial weight 40 g) and 100% survival after 72 days of growth in a self-built low cost recirculation aquaculture system. Those results confirm this fish species can adapt to different

* Illustration. Encyclopædia Britannica Online. Web. 18 Apr. 2018. <https://www.britannica.com/animal/pirarucu?oasmId=4592>

systems without drastic changes in survivability and growth performance, presenting good outcomes for aquaculture production. Moreover, the pirarucu is a fish that tolerates high levels of ammonia and since it breathes air from the water surface it can tolerate low dissolved oxygen levels (Baldisserotto et al., 2008; Cavero et al., 2004; Gonzalez et al., 2010; Pereira-Filho et al., 2003)

Table 1.1: *Arapaima gigas* survival percentage and growth performance in three different aquaculture systems: ponds, cages and recirculation

| System | Initial weight (g) | Final weight (kg) | Survival (%) |
|---|---|---|---|
| Ponds | 15 | 8 to 10 | 90-95 |
| Cages | 500 | 8 to 9 | 90-95 |
| Recirculation | 975 | 10 | 98.7 |

*Data based on fish fed with commercial extruded feed for carnivorous fish (SEBRAE 2010)

**Gut morphology and digestive processes**

Although pirarucu is considered a specialized carnivorous fish, its intestine is medium-long, presenting thick walls and measures around 1.45 times the total length of the fish (Figure 1.3) (Watson et al., 2013). In aquaculture production it responds well for pellet training and accepts easily diets containing 40% of crude protein (Bezerra et al., 2013). The lack of l-gulonolactone oxidase in pirarucu liver and kidney is associated with the inability to synthesize the essential nutrient ascorbic acid (Vitamin C) which its absence is related with scurvy clinical signs (Fracalossi et al., 2001). Likewise, Andrade et al. (2007) confirm that high doses of vitamin C in the diet (800 and 1200 mg $kg^{-1}$) increase red blood cells and white blood cells concentrations that could protect fish under stress. However, few studies have being published about *A. gigas* nutrition requirements and many gaps still need to be filled in terms of micronutrient requirements as well as essential amino acids and protein levels in diet.

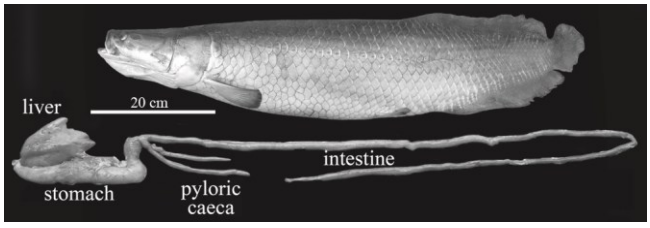



Figure 1.7: Pirarucu, *A. gigas* intestinal tract contents (Watson et al., 2013)

The aforementioned features of the pirarucu (e.g. size, growth etc.) provide

substantial economic promise. The investment made on researching this fish species, follows the concept that farm production reduces fishing pressure on natural populations and allows restocking programs in certain areas (de Oliveira et al. 2012)(de Oliveira et al., 2012). Nevertheless, *Arapaima* production peaked in Brazil was 2014 when 11,763 tonnes were produced; production decreased by 28.7% in 2015 (FAO 2017). This huge drop in production is uncertain. It could be related to disease spread such as parasite infestation or bacterial outbreaks in pirarucu fish farms (Tavares-Dias and Martins, 2017), since there is still a lack of basic knowledge for cultivation of this fish species or economical issues in Brazil during the last years.

## 1.3 Antibiotic use in Aquaculture

Together with the uncontrolled intensification of production, there is a risk and concern about diseases outbreaks due to the lack of specific applied technology for Brazilians native species. As a prophylactic treatment against bacterial diseases many farmers adopt antibiotic use routinely in fish productions (Muñoz-Atienza et al., 2013). The uncontrolled use of antibiotics in fish production can decrease the "equilibrium" of microbiota diversity in fish gut and could eventually increase colonisation by opportunistic bacteria (Romero et al., 2012). Furthermore, the continuous use of antibiotics could lead to the proliferation of antibiotic resistance genes in microbes associated with the fish, environment and also humans through the food chain and those working in the farm environment (Mathur and Singh, 2005).

There is a worldwide concern about antimicrobials used in food production animals, which includes terrestrial and aquatic animals. According to World Health Organization (WHO 2017), the antimicrobial drugs classes such as cephalosporins ($3^{rd}$, $4^{th}$ and $5^{th}$ generation), glycopeptides, macrolides and ketolides, polymyxins and quinolones were considered as critically important and highest priority concern to use in animal production. This classification was assigned due to the fact these antimicrobials are used to treat human infections and also there is evidence of spread of resistant bacteria or resistance genes from non-human sources. However, the use of antibiotics in aquaculture production affects the ecosystem slightly different from terrestrial animals and should be treated as a separated issue.

In the aquatic environment, the genes that decode resistance in bacteria spread more easily through bacterial populations (Romero et al., 2012). Thus,

antimicrobial resistance in bacteria is a high importance topic in aquaculture. However, it is difficult to point one regulation about use of chemicals and antimicrobials in aquaculture since each country has its own regulation for animal production. The U.S.A. Food and Drugs Administration (FDA) Approved Aquaculture Drugs list includes the antibiotics oxytetracycline, florfenicol and sulfadimethoxine (FDA 2017) for use as fish disease treatment or feed additives. On the other hand, the European Union (EU) banned the use of antibiotics as feed additives in animal production in 2006 (EU 2005). Similarly, Brazil has banned the use of several antimicrobial drugs as feed additives by the "*Ministry of Agriculture, Livestock and Supply*" (MAPA), such as chloramphenicol and nitrofurans (IN nº 09, 06/27/2003); tetracycline, quinolones and systemic sulfonamides (IN nº 26, July 9, 2009 that repeals Ordinance nº 193/1998); and spiramycin and erythromycin (IN No. 14, 05/17/2012).

In this context, research into alternative feed additives, as potential substitutes for historically used antibiotics is required. The use of probiotic bacteria in aquaculture is promising and appears to report good results in different species (Banerjee and Ray, 2017).

## 1.4 Probiotic use: benefits and challenges

Between all the probiotic definitions, the classification of Merrifield et al., (2010) was chosen for this work, that being: Probiotics are "microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health". A probiotic must be a complete microorganism that addresses all of those properties. Therefore, in this concept, non-microbial compounds or cellular fractions can be discarded, and microbial cells that are inoculated in the water can be included.

After the definition of probiotics, the wide range of microorganisms can be subclassified (FAO 2016) as follows:

- Bacterial x non bacterial: most of the probiotic used in aquaculture are from bacterial origin, however, some yeast such as *Debaryomyces hansenii* and *Saccharomyces cerevisiae* (Vine et al., 2006) and microalgae *Tetraselmis suecica* (Irianto and Austin, 2002a) were already reported as probiotics in fish.

- Spore forming x non-spore forming: *Bacillus subtilis* and *Bacillus licheniformis* are the most commonly used spore forming strains in aquaculture (Nayak, 2010). The spore-forming characteristic is advantageous since it affords better survival under the adverse conditions during pelleting, storage and upper gastrointestinal tract.
- Multi-strain x single strain: Multi-strain probiotic showed positive results in improving different segments of fish immune response (Aly et al., 2008; Salinas et al., 2005), however, the wide range of available multi-strain probiotic should be better studied due to the fact some strains can antagonised or neutrialised by others and the synergistic effect of a multi-strain probiotic could not appear (Nayak, 2010).
- Autochthonous x allochthonous strains: Autochthonous probiotic bacteria or indigenous probiotic are strains which are isolated from the host, and allochthonous bacteria or exogenous probiotics are generally isolated from a different species or different environment (Nayak, 2010).

It is also important to attest that, amongst many different characteristics a probiont could present during isolation process, many *in vitro* and *in vivo* analyses should be performed to consider a microbe as a potential probiotic. According Merrifield et al. (2010) the microbial organism selection requirements are recorded as essential and favourable characteristics (Table 1.2).

Table 1.2: Probiotic selection requirements: Favourable characteristics and Essential characteristics.

| **Favourable (should)** | **Essential (must)** |
|---|---|
| Be able to adhere/grow in intestinal mucus | Not be pathogenic |
| Be able to colonize intestinal mucosa tissue | Be free of antibiotic resistant genes |
| Be safe to use as feed additive | Resistant to bile salts and low pH |
| Display good growth (microbiological characteristics) | |
| Have antagonistic properties against pathogens | |
| Produce extracellular compounds | |
| Be indigenous to the host or environment | |
| Remain viable under industrial process | |

According to the FAO (2016), the main questions to be focused when assessing a potential probiotic's safety for use in animal feed are: (1) *Does the micro-organism harbour transferable antibiotic resistance gene?* (2) *Is the proposed micro-organism identified to the strain level?* (3) *Is there any evidence of infections associated with the organism?* (4) *Does the micro-organism cause excessive stimulation of the immune system?* Thus, the essential characteristics listed in the Table 2 are rejection characteristics. In other words, if they are positive for pathogenic, resistant genes or negative for bile salts and low pH, the strains should be discarded during screening process. While the favourable characteristics are qualitative, there could be levels or scores for considering a potential probiotic or not. After all *in vitro* and some *in vivo* preliminary assays, microorganims can be described as displaying probiotic potential, and its administration and benefits can eventually be tested *in vivo*.

Among the main benefits reported with probiotic administration are: competitive exclusion of pathogenic bacteria (Vine et al. 2004), the provision of nutrients and digestive enzymes (Mehrabi et al., 2012) and production of antimicrobial compounds such as bacteriocins and organic acids (Balcázar et al., 2007a, 2007b). It is also notable that probiotic bacteria can protect fish against pathogenic bacteria infection due to decreases in mortality after experimental infection (Balcazar et al. 2007b). Therefore, probiotics are not only recognised as potential growth promotors and mucosal microbiota regulators, buy potentially immunomodulators as well (Coppola et al., 2005; Gomez and Balcazar, 2008; Nayak, 2010; Panigrahi et al., 2004).

Fish production companies have a special interest in research about disease resistance and improving immunological efficiency of fish. This reality is not different in Brazil. Some papers studying probiotic selection, and their effects on native South American fish species, in the context of health and nutrition have been published in the recent years. Table 1.3 presents a summary of these published papers. The low number of publications indicates a paucity of research concerning probiotic usage in native Brazilian fish species. Despite this limited research, the results indicate high specificity of the probiotic microorganism and the host, since the microorganisms used in those studies are commonly found as components of the intestinal microbiota of the host in each case.

Some studies have demonstrated that the use of allochthonous bacteria can also provide promising results on fish health (Ridha and Azad, 2012; Standen et al., 2013a); however, there is a general agreement that autochthonous lactic-acid bacterial strains have greater chance to colonize the intestine and bring benefit to the health of the host (Sun et al. 2013). The use of allochthonous strains presents a number of disadvantages. These include: 1) insertion of exogenous microorganisms in the environment, and 3) The ability of these strains to survive or remain in a viable condition and optimal concentrations in the intestinal tract of animals (Nayak 2010).

Table 1.3: Probiotic applications in native South American fish

| **Fish species**<br>**Common name (scientific name)** | **Probiotic species** | **Dosage in diet (CFU $g^{-1}$ of viable counts)** | **Duration probiotic diet** | **Principal findings** | **Reference** |
|---|---|---|---|---|---|
| Angelfish<br>(*Pterophyllum scalare*) | *Bacillus* sp. | *Artemia* sp. enriched with $2 \times 10^7$ | 60 days | >GP<br>>PA *in vitro* | (Dosta et al., 2012) |
| Angelfish<br>(*P. scalare*) | Commercial Pedi-guard® Tehran containing *Pediococcus acidilactici* | *Artemia* sp. enriched with $7 \times 10^9$ | 49 days | > GP<br>> LAB<br>> SR (after salinity stress) | (Azimirad et al., 2016) |
| Surubim hybrid<br>(*Pseudoplatystoma reticulatum* × *P. corruscans*) | *Lactobacillus plantarum* and *Weissella cibaria* | 1 x $10^9$ (+0.5% inulin) | 25 days | > HP<br>> IMP<br>> SR for *W. cibaria* (after *A. hydrophila* challenge) | (Mouriño et al., 2015) |
| Surubim hybrid<br>(*Pseudoplatystoma reticulatum* × *P. corruscans*) | *Weissella cibaria* | 1x $10^6$ | 15 days | Isolation<br>> PA *in vitro*<br>> pHD *in vitro*<br>> HP | (Mouriño et al., 2016) |
| Surubim hybrid<br>(*Pseudoplatystoma reticulatum* × *P. corruscans*) | *Weissella cibaria* | 1 x $10^7$ | 45 days | > HP<br>> IMP<br>> LH, TEM, SEM | (Jesus et al., 2016) |
| Pacu<br>(*Piaractus mesopotamicus*) | Commercial probiotic PAS-TR™ (*Bacillus cereus* and *Bacillus subtilis*) | $1 \times 10^5$<br>$7 \times 10^5$<br>$4.2 \times 10^6$<br>$1.2 \times 10^7$ | 60 days | > IMP<br>> SR (after *A. hydrophila* challenge). | (Farias et al., 2016) |
| Pacu<br>(*P. mesopotamicus*) | Microparticles containing *Lactobacillus acidophilus* | $3.17 \times 10^5$ | 28 days | = GP | (Rodrigues et al., 2014) |
| Tambaqui<br>(*Colossoma macropomum*) | *Bacillus subtilis* | 1.71 x $10^7$ | 60 days | < GP (feed conversion) | (Azevedo et al., 2016) |

| | | | | | |
|---|---|---|---|---|---|
| Silver catfish (*Rhamdia quelen*) | *Bacillus cereus* var. *toyoi* | $5 \times 10^8$ | 30 days | > PA *in vitro*<br>= GP | (Souza et al., 2012) |
| Common snook (*Centropomus undecimalis*) | *Bacillus* sp. | $\cong 10^8$ ml$^{-1}$ (added in water) | 7 days | > GP | (Kennedy et al., 1998) |
| Common Snook (*C. undecimalis*) | *Bacillus subtilis* | $5 \times 10^9$ | 191 days | = GP<br>= PCA<br>= HP | (Noffs et al., 2015) |
| Fat snook (*C. parallelus*) | *Lactobacillus plantarum* | $1 \times 10^7$ | 70 weeks | = GP<br>> HI<br>> HP<br>< TVC<br>< V<br>> LAB | (Barbosa et al., 2011) |
| Fat snook (*C.parallelus*) | *Lactococcus* sp.<br>*Lactobacillus plantarum* | $3.09 \times 10^8$<br>$4.15 \times 10^8$ | 30 days | < V<br>> LAB | (de Souza et al., 2010) |

- Symbol references: > refers to an increase of the analysed parameter; = refers to equal observations of the analysed parameter; and < refers to a decrease of the analysed parameter, when compared to the control.
- Principal findings: GP - Growth performance; LAB - lactic-acid bacteria counts; TVC - total viable counts (total heterotrophic bacteria); V - *Vibrio* spp. counts; HP - haematological parameters; IMP - immunological parameters; SR - survival rate; *A.* - *Aeromonas*; pHD - pH decrease; LH - light histology; SEM - scanning electron microscopy; TEM - transmission electron microscopy; PCA - proximal composition analysis; HI - heparossomatic index.

Seasonality, habitat and age may influence the efficacy of probiotic applications in fish (Merrifield and Carnevali, 2014). In other words, distinctive strains applied to different fish species and variations in water temperatures lead to different bacterial recovery rates in the gastrointestinal tract and also different bacterial interaction with the host. That is the reason species specificity of probiotic and host is so important. For instance, microbiota in Atlantic salmon parr (*Salmo salar*) are influenced by the alteration of environment (loch and closed recirculation aquaculture system) even if the fish were reared in the same condition before being placed into new habitats (Dehler et al., 2017). Thus, the probiotic bacteria that might be effective in one specific environment may not be effective in another. More research is needed in isolation, identification and effects on potential probiotic bacteria for different fish species and consequently different environments.

**Why lactic-acid bacteria (LAB)?**

The Gram-positive bacteria that are able to produce lactic-acid as an end product of fermenetation (Merrifield et al., 2014) are called the lactic-acid bacteria (LAB). They are generally cocci shaped, apart from members of the *Lactobacillus* and *Carnobacterium* genera (Ringø and Gatesoupe, 1998). LAB are aero-tolerant bacteria, a characteristic which explains their ubiquity: they are found in soil, water, associated with plants and intestinal tract of animals (Lauzon and Ringø, 2011). The fact that Qualified Presumption of Safety (QPS) status has been granted by the European Food Safety Authority (EFSA) (Araújo et al., 2016) and their aero-tolerant characteristics are important qualities which have led to the widespread use of LAB as biological agents and probiotics.

von Wright and Axelsson (2011) indicate that LAB are able to ferment carbohydrate (mainly sugar) in different ways. Among them, glucose fermentation is considered the main one. Some strains present homolactic pathways while others are have heterolactic fermentation. Homolactic fermentation bacteria produces only lactate from a glucose molecule, whereas heterolactic fermentative strains produces, apart of lactate, one molecule of ethanol (or acetate) and releases $CO_2$. From galactose for instance, LAB strains can also produce pyruvate by two different pathways. Also LAB are able to cut the disaccharide lactose into glucose or galactose by the lactose-specific system (PEP:PTS) being able then, to enter in the major glucose pathways.

Bacteriocins, produced by LAB and other bacteria, are ribosomally synthesized antimicrobial peptides or bacterial proteins that are able inhibit the growth of other bacteria (von Wright, 2011; von Wright and Axelsson, 2011). Enterococci produce several

bacteroicins, which are called enterocins specifically (Lauková, 2011), and the bacteriocins called lacticins, are produced by lactococci (von Wright, 2011). In general, bacteriocins and other compounds excreted by LAB strains are the main reason they are well studied in terms of fermentation or sugar degradation for food conservation and probiotic utilization for humans and animals.

Between all the species variation of LAB in fish intestinal tract the most common genera include *Streptococcus*, *Enterococcus*, *Lactobacillus*, *Aerococcus*, *Carnobacterium*, *Leuconostoc*, *Lactococcus*, and *Pediococcus* (Ringø and Gatesoupe, 1998), however, the genus *Vagococcus* (Román et al., 2012), *Bifidobacterium* (Vlková et al., 2012) and *Weissellla* (Mouriño et al., 2016) were also isolated from fish. However, it is important to highlight that few LAB are pathogenic for animals and the most notably ones are *Streptococcus iniae* (Wackett, 2016) and *Lactococcus garvieae* (Lauzon and Ringø, 2011). Thus, it is important to go over some characteristics specific for each isolated strain such as favourable and essential characteristics (Table 2) as well as access safety regulations such as EFSA before attest a strain as a potential probiotic.

Although a wide range of LAB were isolated from fish in different environments, (Lauzon and Ringø, 2011) indicated that warm water fish species have higher prevalence of *Lactobacillus*, *Lactococcus* and *Weisella* mainly, but some representatives of *Lactococcus* and *Enterococcus* were also isolated from warm water fish. On the other hand, the genera *Lactococcus*, *Lactobacillus*, *Micrococcus*, *Enterococcus* were listed as being used in freshwater fish such as tilapias, different carp species, zebrafish and catfishes (Dimitroglou et al., 2011). Notably, the potential LAB strains isolated during this thesis, *Enterococcus faecium* and *Lactococcus lactis* subsp. *lactis*, were previously used as probiotics in warm fresh-water fish showing suitable results. For instance, *E. faecium* increased the relative percentage survival (RPS) in common carp (*Cyprinus carpio*) after infection with *Aeromonas hydrophyla* (Gopalakannan and Arul, 2011) and improved weight gain in channel catfish, *Ictalurus punctatus* (Shelby et al., 2007). Immune response was observed in rainbow trout (*O. mykiss*) fed with *E. faecium* (Panigrahi et al., 2007) and colonization of this strain in rainbow trout intestine was attested by Merrifield et al. (2010). Also, tilapia (*O. niloticus*) cultivated in water containing probiotic *E. faecium* presented better growth and immune modulation after 40 days (Wang et al., 2008). On the other hand, *L. lactis* was investigated in tilapia, *O. niloticus*. Positive immunity effects such as higher burst activity, lysozyme content, myeloperoxidase and superoxide dismutase activities were observed after 40 days of probiotic feeding (Zhou et al., 2010). Strains of *L. lactis* subsp. *lactis* appear to present good

results in rainbow trout (*O. mykiss*) and brown trout (*Salmo trutta*). After a short period of 2 weeks, these strains was able to colonize the intestine of rainbow and brown trout as well as improve survival rate after challenge and improve immune responses (Balcázar et al., 2007a, 2007b).

**Modulation of microbiota by LAB**

It has been reported that the gut microbiota in fish is responsible for helping digestion, the production of nutrients and the secretion of antimicrobial compounds that prevent colonization by competitive exclusion of the bacterial pathogens (Nayak, 2010). Several factors that influence/modulate fish gut microbiota such as, host factors (age, sex, species), different diet formulations (lipid, protein and carbohydrates content) and feed additives (probiotics, prebiotics and immunostimulants) were listed by Dimitroglou et al. (2011). Among all the factors that could influence on fish gut microbiota such as sex, genotype or environment, Ghanbari et al. (2015) suggested that diet had the greater than. Thus, the potential variations made by feed additives should follow the same pattern.

The variation of microbiota after probiotic feeding could influence many physiological characteristics of different fish species and it is increasingly being studied (Carnevali et al., 2017). There is reasonable information on fish microbiota modulation after probiotic feeding such as the biological model zebrafish (*Danio rerio*) (Carnevali et al., 2013), Atlantic salmon (*Salmo salar*) (Abid et al., 2013), rainbow trout (*O. mykiss*) (Gisbert et al., 2013), and tilapia (*Oreochromis niloticus*) (Standen et al., 2013b). However, few studies are available in the literature describing intestinal bacterial modulation using the bacteria *Lactococcus lactis* or *Enterococcus lactis* as probiotics in warm freshwater fish species.

Rohu fingerlings (*Labeo rohita*) where fed with *Lactococcus lactis* in combination with *Bacillus subtilis* or *Saccharomyces cerevisiae*, and the three microbes together, for 60 days (Mohapatra et al., 2012). The total heterotrophic bacteria (THB) population was significant lower in all the treatments containing the mix of probiotic listed above. Also, the percentage of *Lactococcus lactis* in gut was near 20% after 15 days of treatment and close to 30% after 30 and 60 days of experiment in each microbial combination. This result is interesting because attests that the strains of *Lactococcus lactis* can possibly be used as multi-strain probiotics with *Bacillus subtilis* and *Saccharomyces cerevisiae*. Likewise, it could indicated that the blend of probiotic micro-organisms in the diet results in better performance in growth performance parameters analysed by Mohapatra et al. (2012). In another study, two

strains of *Lactococcus lactis* spp. *lactis* were also added in Siberian sturgeon (*Acipenser baerii*) (Geraylou et al., 2013) and modulation of fish intestinal microbiota was observed. Bacilli and consequently the genus *Lactococcus* significantly increased in fish fed a *L. lactis* spp. *lactis*. However, no difference in bacterial richness was observed when fish where fed with *L. lactis* strains when compared with control group.

On the other hand, an *Enterococcus* sp. was used by Del'Duca et al. (2013) to supplement tilapia (*Oreochromis niloticus*) diets and showed higher total bacteria counts. Furthermore, it was observed a decrease of counts of pathogens such as and *Pseudomonas fluorescens* and an increase of *Enterococcus* genus in intestinal tract of tilapia. The strain *Enterococcus faecium* (previously *Streptococcus faecium*) improved weight gain, feed conversion ratio (FCR), specific growth rate (SGR) in carp (*Cyprinus carpio*) after 6 weeks of feeding and decreased the Enterobacteriaceae, *Streptococcuss faecalis*, *Staphylococcus aureus* and *E. coli* counts in intestinal tract (Bogut et al., 1998). Similar results were observed by Bogut et al. (2000) in sheat fish (*Silurus glanis*) intestine. There was a decrease of potential pathogenic bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Enterobacteriaceae* and a removal of *Clostridium* spp. after 15 days of feeding with *E. faecium*.

Although, *Lactococcus lactis* and *Enterococcus faecium* strains appear to have positive effect in warm freshwater fish, these papers used culture dependent methods (Mohapatra et al., 2012) and FISH method (Del'Duca et al., 2013) to attest differences in intestinal microbiota. To the author's knowledge, there is no publication analysing the effect of this bacteria in South American native fish using the next generation sequencing (NGS).

Understanding the whole fish gut microbiota is extremely important. Analysing the detailed microbiota at a level to comprehend phylogenetic composition and metabolic potential is essential to understand feed ingredients requirements and essential needs of fish (Ghanbari et al., 2015). Studies on the imbalance in microbiota (dysbiosis) and pathology are well plentiful in humans and land animals. Indeed, it has been suggested that there is a strong relationship between host and their bacterial community (Llewellyn et al., 2014). Although there are several studies being published about fish gut microbiome under many different conditions, there is still a lack of knowledge about the interactions between fish gut microbiome and physiology after probiotic feeding.

NGS approaches has reduced the costs and improved time consumption due to new technologies in this field such as more powerful machines and new bioinformatics methodologies. In fish, NGS studies have being increasing during recent years to help to fill

the gap existent microbial taxonomy and its functional profiling (Figure 1.4) (Ghanbari et al., 2015). The Metagenomics analysis and all its units metatranscriptomics (analysis of transcripts), metaproteomics (expressed proteins), and metabolomics (analysis of produced metabolits); are helping scientists in aquaculture industry to integrate the gut microbiome and develop new strategies in fish nutritional requirements, health status and probiotic use (Ghanbari et al., 2015).

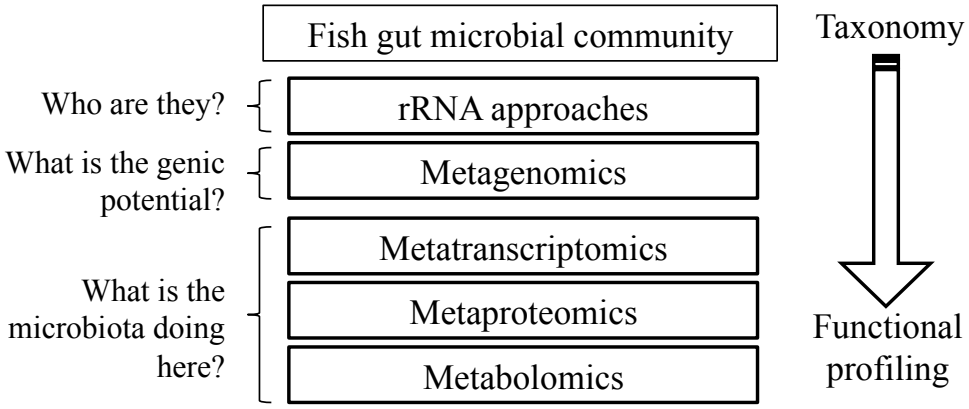



Figure 1.8: NGS approaches from basic amplicon-based to meta-omic to access relationship between fish gut bacteria and fish physiology

The modulation of microbiome was already reported in many fish species using different NGS platforms under distinctive conditions such as: different diet composition in Atlantic Salmon (*Salmo salar*) (Gajardo et al., 2017, 2016), prebiotic supplementation in Siberian sturgeon (*Acipenser baerii*) (Geraylou et al., 2013), captive and wild guppy (*Poecilia reticulate*), and individuals reared in different environments (Wong and Rawls, 2012). However, to the author's knowledge, few studies have been published using NGS tools to assess fish microbiome modulation after probiotic applications.

Standen et al. (2015) used high-throughput sequencing (HTS) to access modulation of microbiota in tilapia (*Oreochromis niloticus*) intestine after addition of a commercial multi-species probiotic AquaStar® Growout. The genera *Bacillus*, *Enterococcus* and *Pediococcus* in gut digesta were significantly higher when compared non-probiotic fed fish. However, the multi-strain commercial probiotic Sanolife PRO-F was not able to modulate intestinal microbiome in tilapia (Adeoye et al., 2016).

Microbial modulation after probiotic bacteria (*Lactobacillus rhamnosus*) feeding in zebrafish (*Danio rerio*) was assessed by HTS (Falcinelli et al., 2017, 2016, 2015). It was observed an increase of Firmicutes abundance and decrease of abundance of Actinobacteria present in intestine of zebrafish after probiotic feeding. Results of lipid content decrease were

related with probiotic supplementation in diet (Falcinelli et al., 2015). Still, in the zebrafish gut *Lactobacillus* abundance was higher in individuals fed a diet supplemented with fed with *L. rhamnosus* (Falcinelli et al., 2016). The same probiotic was also linked with decrease of appetite stimulant related genes and cholesterol and triglyceride related genes (Falcinelli et al., 2017).

Once probiotics are related with host health benefits, more studies accessing gut microbiota and their interaction with fish mucosal immunity, health transcript genes, digestive process and physiological status.

## 1.5 Fish intestinal mucosa immune cells

In teleost, the level of organization of the gut-associated lymphoid system (GALT) is lower than in mammals but is more widespread. GALT comprises a large diversity of lymphoid cells, macrophages, eosinophils and neutrophils (Laing and Hansen, 2011). The presence of intraepithelial lymphocytes (IEL), macrophages and eosinophilic granular cells in the lamina propria in fish intestine (Dalmo et al., 1997) indicate they have a rich and complex immune system. B and T lymphocytes also compose the GALT system in fish; however IEL can be differently distributed through the intestine segments depending of the fish species (Castro and Tafalla, 2015).

The main population of cells in mucosal intestine in European sea bass (*Dicentrarchus labrax*) and common carp (*Cyprinus carpio*) are reported as being T lymphocytes cells (Foey and Picchietti, 2014), and their presence in the intestine can be related with an evolution of mucosal immunity system in fish (Scapigliati et al., 2002). The T cells are involved in cell-mediated cytotoxic activity, humoral immunity and homeostatic responses. T cells have the capacity to receive and recognize a commensal organism to benefit the host, for example probiotic bacteria (Foey and Picchietti, 2014). The increased concentration of T-lymphocytes in the gut of larvae bass (*Dicentrarchus labrax*) induced by probiotic was reported for the first time in fish by Picchietti et al. (2009). Therefore, the use of probiotics in fish diets can modulate the GALT and help the fish to fight against pathogens.

T cells recognize pathogen-associated molecular patterns (PAMPs), expressed by the presence of pathogens, and stimulate B cells proliferation (Foey & Piccietti 2014). The B lymphocytes are the innate defence of the fish while the T-lymphocytes are responsible for responses mediated by other cells, or require a previous stimulus (Magnadottir, 2010). But,

few studies have shown changes of these cells in relation to the feeding of fish with probiotic bacteria present in the diet (Rombout et al., 2011).

Among all the GALT cells, we can highlight also the macrophages, which have a large binding and phagocytic capacities. These cells may carry the antigens for further degradation in the intestinal surface. On the other hand, granulocytes (neutrophils, eosinophils and basophils) generally have the ability to produce and release tryptase, antimicrobial peptides such as lysozyme, piscidina, among others; which are highly related to inflammatory responses in fish intestine (Merrifield & Ringø 2014).

The protection against pathogens of probiotics caused by competition for intestinal wall adhesion and extracellular products released by them are well studied in fish and long established earlier by some authors (Gatesoupe 1999; Merrifield et al. 2010b). However, not much can be said about the true effect on epithelial and dendritic cells in fish when fed diets containing probiotics and or how the released products by the probiotics can modulate the immune system of fish (Rombout et al. 2011).

**Immune cell interactions with probiotics**

Probiotics can interact with cells of the immune system through the gut epithelium, inducing their activity and/or proliferation. For instance, the increase of leucocytes levels in fish blood after being fed with probiotics were observed in tilapia (*Oreochromis niloticus*) fed with *Lactobacillus plantarum* (Jatoba et al. 2011); in rainbow trout fed *Bacillus* and *E. faecium* (Merrifield et al. 2010a) and juveniles of sea bass (*Centropomus parallelus*) fed with *Lactobacillus plantarum* (Barbosa et al. 2011). These findings suggest that in general the probiotics bacteria interfere in fish haematology.

In the studies of Jatoba et al. (2011a) and Barbosa et al. (2011) an increase in peripheral blood lymphocyte concentrations was also reported in fish fed with probiotic strains. The abundance of lymphocytes in the blood may be considered a health indicator, since these cells play important functions in the innate and adaptive defence system in teleost. Other studies have also reported an increase in lymphocytes blood in European sea bass (Picchietti et al. 2009), tilapia (Aly et al. 2008) and rainbow trout (Newaj-Fyzul et al. 2007) after the feeding diets containing probiotics.

Immunoglobulins are produced and secreted by B-lymphocytes and they play an important role in immune teleost fish (Choi and Kim, 2011). They contribute to agglutination, opsonization and activation in complement system (Foey & Piccietti 2014). The level of serum total immunoglobulin in rainbow trout increased significantly after one week feeding

with the probiotic *Lactobacillus rhamnosus* (Nikoskelainen et al. 2003). This result was confirmed by Panagrahi et al., (2005), where supplementation of LAB *Lactobacillus rhamnosus* in the diet, either in live form or in dead form, induced an increase in immunoglobulin concentrations in serum of the rainbow trout. Others authors have also reported an increase of total immunoglobulin in other fish species such as in Nile tilapia (*Oreochromis niloticus*) fed with a mix of *Bacillus* sp. and *Lactobacillus* sp. (Ridha and Azad, 2012); in south American hybrid catfish (*Pseudoplatystoma* sp.) feed with diets containing *Weissella cibaria* (Mouriño et al. 2012); and African catfish (*Clarias gariepinus*) after *Lactobacillus acidophilus* supplementation (Al-Dohail et al., 2009).

It has been reported that the non-specific immune response of fish can increase with feeding of diets containing some probiotics species. Several studies have reported the interaction between probiotic administration and the modulation innate immune responses in fish, for example the phagocytic activity or interaction with polymorphonuclear leucocytes (see review of Nayak, 2010). However, the administration of probiotics should be made carefully, due to the fact some studies stated that the form of administration of the probiotic strains in the diet influences efficacy of the probiotic derived stimulation of phagocytosis (Panigrahi et al. 2005). As described previously, Panigrahi et al. (2009) and Nayak, (2010) reported the increase in phagocytic activity in fish after probiotic supplementation. This increase in head-kidney phagocytic activity was described in rainbow trout fed with different probiotic strains (*Lactococcus lactis* ssp, *Leuconostoc mesenteroides* and *Lactobacillus sakei*) (Balcazar et al. 2007b).

Phagocytic cells such as neutrophils and macrophages play an important role in antibacterial defence, killing bacteria by the production of reactive oxygen species (ROS), including superoxide anion (O2-); hydrogen peroxide ($H_2O_2$); and free hydroxyl radicals (OH-) during respiratory burst (Ellis, 1999). A variety of probioitcs, including the genera *Bacillus*, *Lactobacillus*, *Enterococcus* and *Shewanella* that were administrated as probiotics can increase the burst activity in a lot of fish species attested by review of Nayak (2010). Those fish include Nile tilapia (O. *niloticus*) feed with *B. subtilis* and *L. acidophilus* (Aly et al. 2008) or *Enteroccus faecium* (Wang et al. 2008); rainbow trout feed with *L. rhamnosus*, *B. subtilis* and *E. faecium* (Panigrahi et al. 2007); and in Senegalese sole (*Solea senegalensis*) feed with *S. putrefaciens* (Diaz-Rosales et al. 2009). Conversely, high concentrations of probiotics during long periods in the diet could negatively affect or even decrease the burst activity in fish (Giri et al. 2013). Results of burst activity are controversial because in sole (*Paralichthys olivaceus*) no difference was observed in the respiratory activity when fed

*Lactococcus lactis* subsp. *lactis* (Heo et al. 2013), as well as in gilthead sea bream (*Sparus aurata*) fed diets containing *Lactobacillus delbrueckii* subsp. *lactis* (Salinas et al. 2005) and in Senegalese sole (*Solea senegalensis*) feed with *S. baltica* (Diaz-Rosales et al. 2009). In general, therefore, it seems that high concentrations of probiotics, or long periods of its intake may not be beneficial.

Cytokines are signalling molecules that play a central role in the modulation of immunological and physiological events in animals (Ellis 1999). They are secreted by white blood cells and are an important vehicle for the immune response of fish. The up-regulation of cytokines such as interleukin (IL1-ß), tumor necrosis factor (TNF-α), transforming growth factor (TGF-ß) by leucocytes isolated from the head kidney were observed in the fish fed the probiotic for Panigrahi et al. (2009). The cytokines play an important role in fish immunity in different ways. The most studied interleukin is the IL-ß that is a key mediator against microbial responses (Panigrahi et al. 2007). Other interleukins, such as IL-6, IL-23 and IL-17 mediate anti-fungal responses (Foey & Piccietti 2014). The TNF cytokines are involved in the development functions of the Th1 cells that drive response to intra-resident pathogens; and TGF-ß is related with tissue remodelling and sore repair (Panigrahi et al. 2007). The expression of cytokines genes related with immunological response in fish was reported in several studies such as in common carp (*Cyprinus carpio*) fed with yeast extracts (Biswas et al. 2012); in Japanese pufferfish (*Takifugu rubripes*) fed with *Lactobacillus paracasei* subsp. *paracasei* and *L. plantarum* (Biswas et al. 2013); in rainbow trout fed with three different diets containing the LAB *Lactobacillus rhamnosus*, *Enterococcus faecium* and *Bacillus subtilis* (Panigrahi et al. 2007); and in olive flounder (*Paralichthys olivaceus*) fed with *Lactococcus lactis* and *Lactobacillus plantarum* (Beck et al. 2015). In general, therefore, it seems that the probiotic help to regulate and increase the basic innate components of fish immune responses.

Within the humoral responses, lysozyme is an important bactericidal enzyme of the immune system and has Gram-positive bacterial specificity since it acts on peptidoglycan (Giron-Perez et al. 2009). Increased serum lysozyme activity was observed by Merrifield et al. (2010a) in rainbow trout fed diets containing *Bacillus* probionts and was confirmed also by Panigrahi et al. (2009). However, Mouriño et al. (2012) did not detect differences in the concentration of serum lysozyme in surubim hybrid *(Pseudoplatystoma corruscans* x *P. fasciatum*) fed with *Weissella cibaria* compared with fish did not receive probiotic in the diet, as well as Wang, et al. (2008) that did not find difference in the concentration of lysozyme in Nile tilapia fed probiotic *Enterococcus faecium*. These results highlight that the use of

probiotics is not an easy topic and the modulation of immune system can be influenced by several factors such as, concentration of probiotic, species of probiont and the time of probiotic intake as well.

The enzyme myeloperoxidase is released by neutrophils and it acts to destroy pathogens by the production of oxidative radicals (Dalmo et al. 1997). Recently, Das et al. (2013) reported that the supplemented diet with the probiotic *Bacillus amyloliquefaciens* increase the serum myeloperoxidase concentration in catla (*Catla catla*) after 4 weeks feeding with the probiotic bacteria.

Teleost have three pathways in complement system: the alternative complement pathway (ACP), the classical complement pathway (CCP) and the lectin pathway (Balcazar et al. 2007b). The ACP activity is very important in fish and is activated by the detection of bacteria cell wall lipopolysaccharide resulting in its break. On the other hand, antibodies bounded to the wall of the bacteria activate the CCP, and then a larger amount of the complement system can be activated in comparison to the ACP. The antibodies can even mark sites for the CCP in the bacterial wall where the complement system may cause greater damage to the bacteria (Ellis, 1999). The lectin pathway is activated by specific lectins such as mannose-binding lectin (MBL) and ficolins. They act as opsonization of phagocytic activity, they play an important role in the activation of complement system, and involve carbohydrate recognition present on pathogens cellular wall (Boshra, Li & Sunyer 2006; Fujita, Matsushita & Endo 2004). Consequently, when evaluating fish were fed with probiotic would be interesting to observe further the main differences in the alternative complement system ACP.

It was reported that brown trout (*Salmo trutta*) (Balcazar, et al. 2007a) and rainbow trout (Balcazar et al. 2007b) fed with LAB demonstrated increased serum activity of complement system ACP after two weeks of probiotic supplementation. This increase in the ACP have also occurred in the grouper (*Epinephelus coioides*) fed with diet containing *B. subtilis* at a concentration of $10^6$ and $10^8$ CFU $mL^{-1}$ for 14 days. This same work reported that grouper fed with the same bacteria have also increased ACP at a lower concentration ($10^4$ CFU $mL^{-1}$); however the effects in ACP appeared only after 28 days. These results confirm that the concentration of probiotic bacteria influences the response of the complement system, as well as supplementation of such time (Liu et al. 2012).

## 1.6 Hypothesis and aims

There are several prophylactic treatments in aquaculture to avoid the use of antibiotics to control or prevent disease from virus and bacteria in aquaculture such as immune stimulants, vaccines, feed additives and water treatments. Among them, the use of probiotics in aquaculture production is well known as beneficial to the fish helping themselves to improve several physiological conditions.

The use of gut autochthonous bacteria as probiotics in fish diets has shown promising results when it is evaluated growth performance, gut modulation and immunomodulation. Therefore, the isolation of a probiotic bacterial from the own *Arapaima gigas* (pirarucu) gut presents promising. There is a lack of knowledge about pirarucu fish and naturally about its gut microbiota, potential probiotic bacteria and its application for this emerging species. Thus, the main hypothesis of this thesis is, if the authochtonous probiotic, could firstly be isolated, and then if it could help the *A. gigas* to improve since growth performance until punctual immunologic responses.

In this context, the central objective of this thesis is to select an indigenous probiont organism that can be introduced in the *A. gigas* production in Brazilian farms with the objective of prevent bacterial diseases in pirarucu culture and support the sustainable culture of this fish and the development of research in South-American native fish. For that propose, the general and specific aims for this thesis are as follows:

- **General**

Contribute to the development of research and extension focusing on the use of probiotics for Brazilian native fish in order to prevent diseases of bacterial origin in farms growing *Arapaima gigas*.

- **Specific**

a) To isolate and identify lactic acid bacteria (LAB) from pirarucu intestine with probiotic potential focousing on charachteristics such as absence of antibiotic resistance genes for antibiotics generally used in aquaculture farms, viabitity in fish diets and abstence of pathogenic records in the literature.

b) To isolate and identify possible bacterial etiologic agents during mortality outbreaks by setting up a challenge experiment to assess the lethal dose concentration (LD50) for the *Arapaima gigas.*

c) To assess *in vitro* LAB strains with greater potential probiotic for the inhibition of bacterial pathogens for aquaculture using the agar disk diffusion antagonism technique.

d) To evaluate the colonization of the intestinal tract of fish fed diets containing the probiotic bacterial strain selected in the previous step by administrating the potential probiotic bacteria to *A. gigas* diets.

e) Perform a growth experiment to compare the effects probiotic administration in diets on microbiological, haematological, immunological and zootechnical parameters in *A. gigas*.

# Chapter 2:
# General Material and Methods

# Chapter 2: General Material and Methods

All laboratory analyses were undertaken at Plymouth University following standardized protocols and all *in vivo* experiments were undertaken in Brazil due to fish logistical constraints. The experimental infection (LD 50) was carried out at the AQUOS (Aquatic Organisms Health Laboratory) facilities, which belong to the Aquaculture Department of the Federal University of Santa Catarina (UFSC) Brazil. The two *in vivo* experiments, which follow, were undertaken in the fish farm *Mar & Terra Ind. Com. de pescados S/A* facilities in Pimenta Bueno city located in Rondônia state, Brazil. The fish initial weights, acclimation periods and water quality conditions during each experiment are explained in the corresponding chapters of this thesis.

## 2.1 AQUOS facilities

The experiment was carried out in a room where there were 24 tanks with 100L capacity. The tanks were maintained in a closed-water recirculating system with mechanical and biological filters under a constant temperature of 28°C. The water quality parameters were monitored daily. When necessary, 20% to 60% of the water in the system was replaced.

## 2.2 *Mar & Terra* facilities

The "Reproduction of Amazonian Native Fish" unit from *Mar & Terra Ind. Com. de pescados S/A* is one of the few Brazilian farms authorized to produce Pirarucu (*Arapaima gigas*). In the juvenile fish production facility, there were 18 tanks (1000 l). The tanks were placed in a flow-through system fed by a main weir. The main weir provides the water for the farm by gravity, passing first through early stage fishponds until finally reaching the breeding fish.

## 2.3 Experimental approach

For the isolation of potential autochthonous probiotic bacteria from pirarucu (*Arapaima gigas*), the experimental design of this thesis was as divided in two sections: 1) Isolation and *in vitro* analysis, and 2) *in vivo* experiments. Figure 2.1 represents a summary of the experimental approach. For detailed methodology, read the following specific sections.

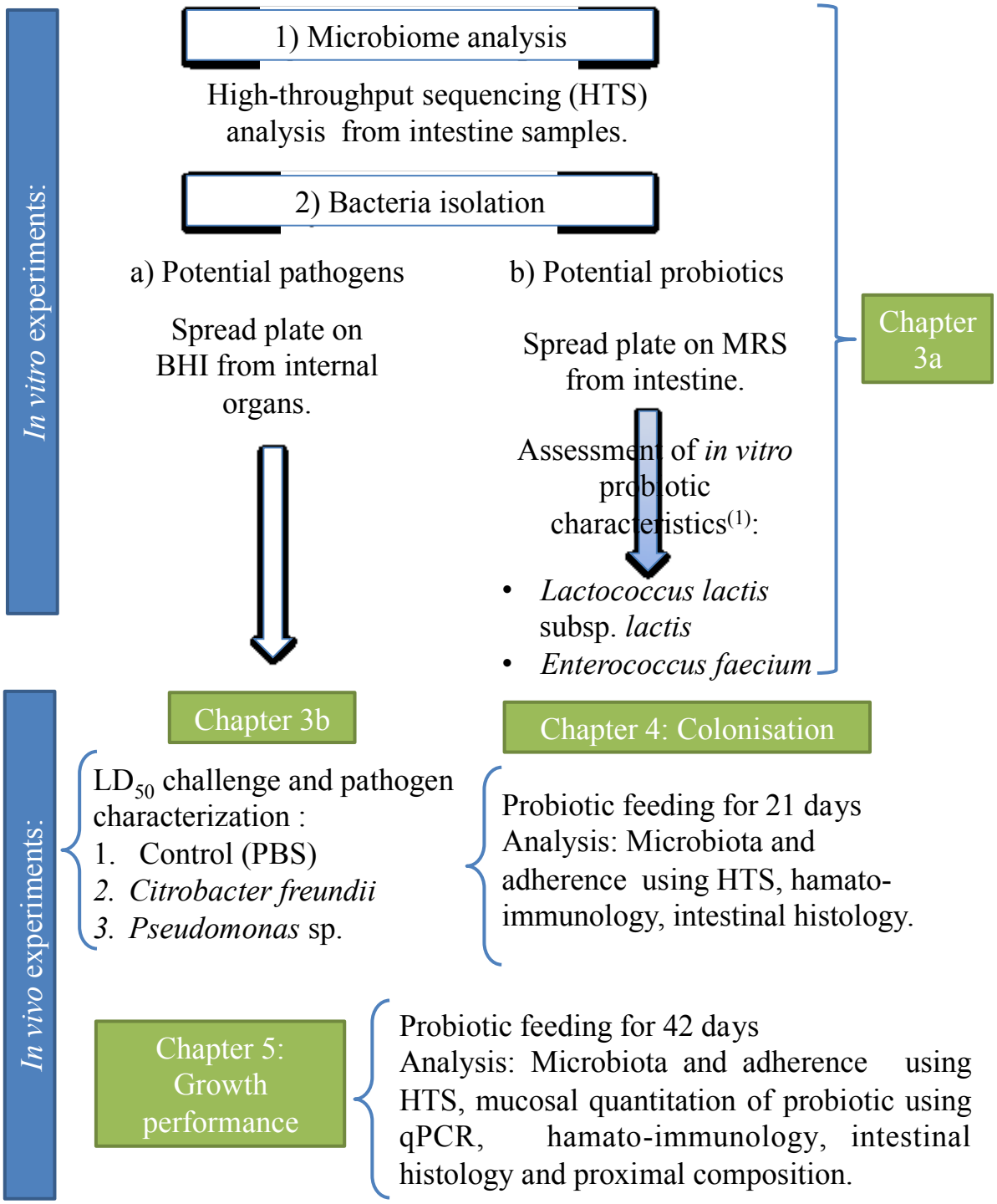



Figure 2.1: Overview of the experimental approach used and mapping against the relevant chapters. (1) Potential probiotic characteristics analysed: absence of antibiotic resistance characteristics, pathogen antagonism, absence of haemolytic activity, and viability in fish diets

**2.4 Isolation of potential probiotic bacterial strains**

Nineteen healthy *A. gigas* in three different life stages were euthanized and sampled from the *Mar & Terra Ind. Com. de pescados S/A*: three fish from post-hatch stage (1.5 cm); five hatchery fish (8 cm); and eleven adult fish (150 cm). The intestines were aseptically

excised and rinsed with sterile saline solution (0,65% NaCl, Synth Sao Paulo). The tissue was inoculated in de Man Rogosa and Sharpe (MRS, Difco Sao Paulo) broth and incubated overnight at 37 º C. The inoculum was then spread onto MRS agar plates with 1% of aniline blue 1% (v/v) and incubated at 35 ºC for 48 h. The colonies that presented lactic-acid production (identified by a blue coloration) were isolated on new MRS agar plates and stored for further molecular identification. A total of 40 isolates were selected and analysed morphologically by Gram staining. After which, 23 Gram-positive strains with bacilli, coccobacillus and cocci morphology were sub-cultured for DNA extraction and identification by 16S rRNA.

**2.5 Isolation of potential pathogenic bacterial strains**

Five moribund *A. gigas* of approximately 8 cm length, from the *Mar e Terra Ind. Com. de pescados S/A* were sampled during a previous, isolated, disease outbreak period. The fish were anesthetized with 0.01% eugenol (Vetec/Sigma-Aldrich) and euthanized. Then the liver, kidney and heart were excised and homogenised in a sterile mortar and pestle with 0.65% NaCl solution at the ratio of 1:1. The tissue homogenates were then spread on TCBS agar (Himedia®, Mumbai, India); KF *Streptococcus* agar; and Cetrimide agar (Himedia®, Mumbai, India). Resulting isolates were subcultured and retained in BHI medium (Merck®). Subsequently, the strains were biochemically and molecularly identified by 16S rRNA sequencing.

For biochemical tests API 20E V5.0 (BioMérieux, Marcy l'Etoile, France) were used for identification of *Enterobacteriaceae* and/or non-fastidious Gram-negative rods. The pure colonies were prepared according to the manufacturer's instructions and inoculated in each well of the API strip containing 21 different ingredients. The change of color for each specific cup gives a +/- combination that corresponds with a numerical profile, which values are translated to species identification.

**2.6 DNA extraction from pure colonies**

The LAB bacteria were grown overnight in MRS broth at 37 ˚C and pathogenic strains in BHI broth at 30˚C, centrifuged at 4,000 g for 10 min and re-suspended (0.5% times of the original volume) in TE buffer (Tris-EDTA Sigma-Aldrich). The DNA was extracted from the pellets using the CTAB method, adapted from the Joint Genomic Institute (JGI) protocol (William and Copeland 2004). In brief, 100 µl of freshly made lysozyme (100 mg.ml $^{-1}$ TE Buffer (Tris-EDTA Sigma-Aldrich)) was added to 500 µl of the bacterial cell

suspension and incubated at 37˚C for 30 min for cells lysis and, 10% SDS and 10 µl of proteinase K (20 mg $ml^{-1}$ Qiagen, Germany) was added before an additional incubation for 30 min at 56˚C for protein removal. After the addition of 120 µl of 5M NaCl and 120 µl of warm (56˚C) of CTAB/NaCl the tubes were incubated for 10 min at 56˚C. For cleaning, 460 µl of Tris-Buffered phenol (Sigma-Aldrich) was added and the tubes left stand for 10 min in ice. The same volume of chloroform (Sigma-Aldrich) was added and the tubes centrifuged for 5 min at 6,000 g. The upper layer was carefully removed and placed in a new tube. An amount equivalent to 0.8 of the total volume of ice-cold isopropanol (Sigma-Aldrich) was added in the tube containing the samples and left in ice for 10 min to precipitate the DNA. Afterwards, the tubes were centrifuged up at 14,000 g for 10 min to pellet the DNA. The supernatant was then removed and the pellets washed with 1 ml of 70% molecular grade ethanol (Sigma-Aldrich). The samples were centrifuged again under the same conditions and left to dry close to Bunsen burner for 5 min before being dissolved in 30 µl of TE $^{-1}$ Buffer for storage and additional PCR analysis.

**2.7 DNA extraction from mucosa and digesta samples**

All mucosa (intestinal tissue) and digesta (intestinal content) were stored in DNA free vial tubes with sterile molecular grade ethanol 98% (Sigma-Aldrich). Prior extraction, samples were centrifuged (17.000 g for 5min) and ~200 mg of each sample was weighed under aseptic conditions. The DNA was extracted using QIAamp Stool Mini Kit (Qiagen, Germany). Prior to extraction, the samples were mixed with 500 µl of freshly made lysozyme at (500 mg.ml $^{-1}$ TE Buffer) and incubated for 30 min at 37˚C in order to increase the disintegration of gram positive bacteria by rupture of the cell wall. Then 700 µl of ASL Buffer was added to each tube, which were mixed and heated for 5 min at 95˚C. The tubes were then centrifuged for 1 min at 13,000 g. The supernatant (~200 µl) was removed, placed in a new tube containing inhibitex tablets and centrifuged for 3 min. For protein removal, 15 µl of Proteinase K was added to 200 µl of the supernatant and 200 µl of AL Buffer and incubated at 56˚C for 30 min. After the incubation period 200 µl of 98% ethanol was added and the content applied to a QIAamp column and centrifuged for 1 min. The columns were washed twice with 500 µl of each Buffer AW1 (for 1 min) and AW2 (for 3 min). The DNA was finally eluted in 30 µl of Buffer AE after 1 min centrifugation of the columns. The eluted DNA was then placed into a DNA free Eppendorf® and stored at -20˚C for future analysis.

**2.8 PCR for molecular identification of pure colonies**

The extracted DNA was used as template for PCR using the primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) for 16S rRNA gene identification (Hagi et al., 2004). The 25 µl reaction mixture consisted of 12.5 µl Taq (Mytaq Red Mix Bioline® Ltd), 10.5 µl of autoclaved molecular water, 0.5 µL of each primer (50 pmol.$\mu l^{-1}$) and 1.0 µl template. For the positive control 1.0 µl of extracted DNA from *E. coli* was used and for the negative control the same volume of sterile molecular water was used. The PCR thermal cycling GeneAmp® PCR System 9700 machine and the conditions used were: 9 min preheat at 95˚C, and 35 cycles of 1 min of denaturing at 95˚C, 1 min for primer annealing at 53˚C and 2 min for primer extension at 72˚C.

**2.9 PCR for High throughput screening**

To perform high-throughput sequencing analysis the extracted DNA from mucosa and digesta samples were used as templates for PCRs using primers 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and, pooled 338R-I (5' GCW GCC TCC CGT AGG AGT 3') and 338R-II (5' GCW GCC ACC CGT AGG TGT 3'). The 50 µl reaction PCRs were performed using 1.0 µl of template (approximately at 50 ng. $\mu l^{-1}$), 25 µl of MyTaq™ Red Mix (Biolone, London, UK), 0.5 µl of each primer (at 50 pmol.$\mu l^{-1}$), and autoclaved molecular water to complete the reaction volume. Thermal cycling was conducted in a GeneAmp® PCR System 9700 machine. The conditions used were: initial denaturation at 96˚C for 3 min, and 35 cycles of denaturation at 94˚C for 15s. A touchdown annealing strategy was used as follows: 10 cycles decreasing from 63˚C to 53˚C for 30s followed by 25 cycles at 53˚C for 30s, and extension at 72˚C for 30s.

**2.10 Agarose gel electrophoresis**

The quality of the PCR products was checked using agarose gel electrophoresis. An aliquot of 8 µl of each sample was added to wells in a 1.5% agarose gel in TAE Buffer (Biolone, London, UK) (1x Tris acetate EDTA) contaiing SYBR Safe DNA stain (1 µl per 10 ml of still warm agarose gel). Eight µl of Hyper ladder IV (Biolone, London, UK) was run in each gel. The gel was then placed in an electrophoresis tank (Biometra®, Germany) and run for 40 min at 90 V.

**2.11 16S rRNA sequencing**

Before sequencing the PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Germany) and then sent to sequence by GATC laboratories

(Germany). The resultant nucleotide sequences were submitted to a BLAST search in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to retrieve the closest known alignment identities for the partial 16S rRNA sequences. Species level identification was accepted at ≥97% nucleotide alignment.

**2.12 High throughput screening (HTS)**

PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Germany) and quantified using a Qubit® 2.0 Fluorometer (Invitrogen). Before sequencing the amplicons were assessed for fragment concentration as described by Falcinelli et al. (2015). All taxonomic analyses were performed after the removal of low quality scores (Q < 20) with FASTX-Toolkit (Hannon Laboratory, USA). Sequences were concatenated and sorted by sequence similarity into a single fasta file, denoised and analyzed using QIIME 1.8.0 (Caporaso et al., 2010b). The USEARCH quality filter pipeline (Edgar, 2010) was used to filter out putative chimeras and noisy sequences and carry out operational taxonomic unit (OTU) picking on the remaining sequences. The taxonomic affiliation of each OTU was determined based on the Greengenes database (DeSantis et al., 2006) using the RDP classifier (Wang et al., 2007) clustering the sequences at 97% similarity with a 0.80 confidence threshold and a minimum sequence length of 300 base pairs. Non-chimeric OTUs were identified with a minimum pairwise identity of 97%, and representative sequences from the OTUs were aligned using PyNAST (Caporaso et al., 2010a). To estimate bacterial diversity, the number of OTUs present in the samples was determined and a rarefaction analysis was performed by plotting the number of observed OTUs against the number of sequences. Additionally, QIIME was used to assess Alpha diversity parameters including Good's coverage, Shannon-Wiener (diversity) and Chao1 (richness) as well as Beta diversity parameters using weigthed and unweigthed Unifrac distances.

**2.13 Antibiotic resistance**

The extracted DNA from LAB was also used for the detection of ARGs by PCR. A number of genes conferring resistance to erythromycin (*erm*(A), *erm*(B), *erm*(C)), tetracycline (*tet*(M), *tet*(L)) and chloramphenicol (*cat*) were assessed. The primers were previously described by Toomey et al., (2010) and the amplifications conditions were followed according previous publications (Table 2.2).

Table 2.2: List of genes conferring resistance to erythromycin (*erm*(A), *erm*(B), *erm*(C)), tetracycline (*tet*(M), *tet*(L)) and chloramphenicol (*cat*)

| Gene | Primers (5'-3') | PCR conditions reference |
|---|---|---|
| *tet*(M) | Fwd: GTG GAC AAA GGT ACA ACG AG<br>Rev: CGG TAA AGT TCG TCA CAC AC | Ng et al. (2001) |
| *tet*(L) | Fwd: TCG TTA GCG TGC TGT CAT TC<br>Rev: GTA TCC CAC CAA TGT AGC CG | Ng et al. (2001) |
| *tet*(W) | Fwd: GAG AGC CTG CTA TAT GCC AGC<br>Rev: GGG CGT ATC CAC AAT GTT AAC | Aminov et al. (2001) |
| *erm*(A) | Fwd: TCT AAA AAG CAT GTA AAA GAA<br>Rev: CTT CGA TAG TTT ATT AAT ATT AGT | Sutcliffe et al. (1996) |
| *erm*(B) | Fwd: GAA AAG GTA CTC AAC CAA ATA<br>Rev: AGT AAC GGT ACT TAA ATT GTT TAC | Sutcliffe et al. (1996) |
| *erm*(C) | Fwd: TAC AAA CAT AAT ATA GAT AAA<br>Rev: GCT AAT ATT GTT TAA ATC GTC AAT | Sutcliffe et al. (1996) |
| *cat* | Fwd: TTA GGT TAT TGG GAT AAG TTA<br>Rev: GCA TGR TAA CCA TCA CAW AC | Hummel et al. (2007) |

Antibiotic minimum inhibitory concentration (MIC) for LAB was also assessed using VetMIC$^{TM}$ Lact-1 microplates (National Veterinary Institute, Uppsala, Sweden). The antibiotics included in the microplate were gentamycin, kanamycin, streptomycin, neomycin and tetracycline, erythromycin, clindamycin and chloramphenicol and the assays were conducted according to the methodology described by Munoz-Atienza et al. (2013). MICs were accepted as the lowest antibiotic concentration that inhibited bacterial growth, and interpreted according to the breakpoints approved by EFSA (2012).

### 2.14 *In vitro* antagonism of fish pathogenic bacteria by potentially probiotic strains

The antagonistic properties of the LAB isolates, against the pathogenic strains, was evaluated in triplicate, using the agar disk diffusion antagonism technique as described by Vieira et al. (2007). Briefly, the isolated LAB strains were plated on MRS agar at the log phase and incubated for 48 h at 37 ºC. After the incubation period, 0.8 cm diameter agar discs were taken from these plates. Six pathogenic strains isolated from moribund *A. gigas* (isolated in Chapter 3 of this thesis), and standard fresh water pathogenic strains: two strains of *Aeromonas hydrophila* (DRM CPQBA 228-08 isolated from hybrids surubim, and ATCC 7966 isolated from Nile tilapia), one strain of *Micrococcus luteus* (A270) and one of *Streptococcus agalactiae* (GRS2035), both isolated from Nile tilapia, were grown in BHI medium (Oxoid, UK) for 24 h at 30 ºC. After incubation, 1:10 serial dilutions were performed to achieve the concentration of $10^5$ CFU.ml$^{-1}$, then, the bacteria were plated on

Mueller-Hinton plates (Oxoid, UK) with glucose (3 $g.l^{-1}$). The agar discs containing LAB were placed on the agar surface, followed by incubation for 24 h at 30ºC. Antagonistic activity was defined by the diameter (cm) of the clear inhibitory zone formed around the disc.

**2.15 Haemolytic assay**

Fresh colonies for all LAB stains were plated on triplicate blood agar plates, containing 5% (w/v) sheep blood (Fisher Scientific, Madrid), and incubated for 48 h at 37ºC. Blood agar plates were examined for signs of β-haemolysis (clear zones around colonies), α-haemolysis (green-hued zones around colonies) or γ-haemolysis (no zones around colonies).

**2.16 Motility**

One fresh colony, from each bacterial strain identified as potentially pathogenic, was inoculated stabbing the middle of the emi-solid PB agar (Poor Broth medium, 1% peptone, 0.5% NaCl and 0.5% agar) medium. The positive movement of the bacteria through the medium generates a diffuse zone of growth spreading from the line of inoculation.

**2.17 Viability of LAB in high protein diets**

The selected LAB were evaluated for viability in fish. The strains were grown in MRS culture medium (Oxoid, UK) at 37°C for 24 h and then centrifuged at 4,000 g for 20 min, re-suspended in PBS followed by serial dilutions in PBS to obtain a concentration of $1x10^{9}$ $CFU/ml^{-1}$. The bacterial suspension was uniformly sprayed onto a commercial diet (BioMar Efico Enviro) at 100 $ml.kg^{-1}$. The diet was subsequently dried at 50 °C for 3 h and then stored at 4 °C. The diet composition according the manufacturer was: 42-45% crude protein; 27-30% crude lipid; 16% carbohydrates (NFE) and 1.7% of fiber. The concentrations of LAB were quantified by plating out dilutions (1:10) of 1g of diet, in triplicate, on MRS plates with 1% aniline blue and incubated 37 °C for 24 h. The concentrations of the microorganisms were measured in colony forming units per g (CFU $ml^{-1}$) each week for four consecutive weeks.

**2.18 Inoculation of probiotic bacteria**

The extruded commercial diet *Do Peixe Revolution Alevino 2 to 3 mm* (Douramix, Brazil) which comprised of 40% crude protein, and 11% crude lipid was used during the two *in vivo* feed experiments in the fish farm.

Both probiotic bacteria *L. lactis* subps. *lactis*, and *E. faecium* were grown in MRS broth (Oxoid, UK), and incubated at 35°C for 48h. The suspension was centrifuged at 4,000 g for 20 min and re-suspended in PBS (Oxoid, UK). One hundred mL of the probiotic at the

desired concentration ($1x10^9$ CFU $mL^{-1}$ or $1x10^8$ CFU $mL^{-1}$) was sprayed, using a sterile plastic spray bottle, on each kilogram of the commercial diet. The diet was dried at 30°C for 12h. This process was repeated every week to accomplish the same probiotic concentration in the diet.

To determine the probiotic concentration in the diet, 1g of the diet with probiotic was macerated in a sterile mortar with 9 ml of PBS and then serially ten-fold dilutions were conducted. The dilutions $10^{-5}$ to $10^{-9}$ were spread plated on petri dishes containing MRS agar with 1% of aniline blue 1% (v/v). The plates were incubated at 35°C for 48h. This process was repeated every time the diet with probiotic was prepared.

**2.19 Haematology**

The blood was collected by puncture of the caudal vessel. The collected blood was used to make blood smears stained with Giemsa/ May Grunwald staining (Rosenfeld 1947) for differential leukocyte counts. An aliquot was used to determine the haematocrit (Goldenfarb, Bowyer, Hall & Brosious 1971) and 20µl was diluted in 980µl Dacies solution to quantify the total number of erythrocytes (RBC) by Neubauer chamber.

**2.20 Immunology assays**

Blood samples were left overnight at 4 °C to coagulate prior to centrifugation at 1400 g for 10 min to obtain the serum which was stored at -20°C.

The serum lysozyme was determined using the methodology adapted by Ellis (1990). A volume of 20 µl of serum, in five replicates, was inoculated into flat bottom microplate, and 200 µl of *Micrococcus lysodeikticus* (Sigma-Aldrich, USA) (0.02% (w/v) in PBS) was added to each well. The initial and final absorbance (after 10 min at 35 °C) of the samples were measured in a microplate reader (Expert Plus Asys®) at 492nm and the rate of reduction in absorbance of the samples was converted to lysozyme concentration (µg $mL^{-1}$) determined by a standard curve previously made with lysozyme from chicken egg whites (HEWL; Sigma-Aldrich).

Serum bactericidal activity was tested against *Pseudomonas* sp. and *Citrobacter freundii* (isolated in chapter 3). Both strains were grown in BHI (Oxoid, UK) at 30 °C for 24 h and then diluted in PB (Poor Broth medium, 1% peptone and 0.5% NaCl) to a final inoculum concentration of $1x10^5$ CFU.$ml^{-1}$. The antimicrobial activity was tested in one 96-well microplate for each strain. A volume of 100 µl of serum was poured into the first well of the plate and 12 serial two-fold dilutions using PB were performed in the remaining wells of a row. For positive and negative controls, sterile saline solution (0,65% NaCl, Synth Sao

Paulo) was diluted in PB in the same way. Finally, 20 µl of the inoculum was added to each well of diluted serum and positive controls. Negative control remained sterile (no added inoculum). The microplates were covered with a lid and incubated at 30°C for 24h. The bacterial growth was determined by measuring the absorbance at 550 nm using a plate reader (Expert Plus Asys®). The last serum dilution that showed bacterial growth when compared to positive control was used as estimator of serum bactericidal activity.

**2.21 Histology**

A portion of 5 mm from posterior intestine were sampled and fixed in 10% formalin for 76 h and then placed in 70% ethanol for storage. Samples were then treated with 10% formal saline for 5 min; dehydrated in a graded ethanol series (50% IMS for 2.5 h, 70% IMS for 2.5 h, 90% IMS for 2 h, 100% IMS for 2h, 100% IMS for 1.5 h, 100% IMS for 1.5h); filled with wax (2x wax for 2h) and embedded in paraffin wax using a Leica EG1150H.

Three to four sections of 3 µm from each sample (wax block) were cut using a microtome (Leica), placed onto glass slides and left to dry overnight. The slides were then staining using hemayoxilin and eosin (H&E) protocol and Alcian Blue-PAS (AB-PAS) protocol.

For H&E the slides were stained following Howard et al. (2004). The slides were hydrated with water and stained with Harris' hematoxylin for 2 min, washed in 0.5% lithium carbonate for approx. 30 sec, then placed in 70% ethanol for 2 min and counterstain for 1 min in eosin. For AB-PAS, the slides were hydrated in histolene for 5 min and in 100% IMS for 5 min, placed in 1% Alcian Blue for 20 min, oxidized with 1% Periodic Acid for 10 min, placed in Schiff reagent for 3 min, stained lightly with Haematoxylin for 90 sec and blue in Lithium Carbonate <10 seconds. Between each change of reagent the slides were washed with running tap water. After that, the slides were placed in 100% IMS and histolene for 1 min in each one, coverslipped using DPX and left to dry overnight.

Photographs were taken using Leica digital micro-imaging device (DMD108, Leica Microsystems) and analyzed using imageJ v1.4r. A total of 6 to 8 folds were analyzed per sample. Three different goblet cells: acidic mucins (bright blue), neutral mucins (magenta), and both acidic and neutral mucins (blue-purple or purple) were observed and quantified within a distance of 100 um from the top of each fold. The LP width and fold length was also measured for each sample. For the LP width, three measurements (bottom, middle and top) were recorded and the average of them was annotated as one fold measurement. The "segmented line" from imageJ softweare was used to measure fold length.

## 2.22 Identification of pathogenic bacteria in tissue

A section of 1g of the liver from the surviving fish was macerated in a sterile mortar with 9 ml of PBS and then serially diluted in vials tubes at 1:10 factor. The dilutions $10^{-1}$ to $10^{-8}$ were spread plated in petri dishes containing TSA agar (Himedia®, Mumbai, India) and Cetrimid agar (Himedia®, Mumbai, India). The plates were incubated at 28°C for 48h. The total counts for both media were noted and the colonies analysed morphologically by Gram staining under microscope. Different colonies were then streaked onto the same media to assure purity. The pure colonies were inoculated in BHI Broth medium, incubated for 24h at 28°C. The DNA from the colonies was extracted using the same protocol described in session 2.6 of the present chapter. The extracted DNA was used as template for PCR using the primers 27F and 1492R (see session 2.7).

## 2.23 Growth analysis

The initial weight (IW), final weight (FW) as well as the initial length (IL) and final length (FL) of each fish were recorded at the beginning and end of the experiment, respectively. The calculations for net weight gain (NWG), percentage of increase (%I), feed intake (FI), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER) and condition factor (K) were conducted using the following formulae:

- $\mathrm{NWG} = \mathrm{FW} - \mathrm{IW}$
- $\%\mathrm{I} = \left(\left(\frac{100}{\mathrm{IW}}\right).\mathrm{FW}\right) - 100$
- $\mathrm{SGR} = 100\ \left(\frac{\ln \mathrm{FW} - \ln \mathrm{IW}}{\mathrm{T}}\right)$
- $\mathrm{FCR} = \mathrm{FI}/\mathrm{WG}$
- $\mathrm{PER} = \mathrm{WG}/\mathrm{PI}$
- $\mathrm{K} = \frac{(100\ .\mathrm{FW})}{\mathrm{FL}^3}$

Where T = duration of feeding (days), FI = feed intake, PI = protein ingested. All parameters were evaluated in grams.

## 2.24 Carcass composition

Three fish per experimental unit were sample for carcass composition. For moisture, ash, protein and lipid, the AOAC (2005) protocol was followed. All samples were analysed in triplicate.

### 2.24.1 Moisture

For moisture content, fish wet were weighted and placed into an incubator for 72 hours at 105°C. The dry samples were then weighted and the difference between dry and wet fish was accounted as moisture in each sample following the equation:

$$\%\ Moisture = \frac{(Wet\ weigth - Dry\ weigth)}{Wet\ weight}\ x\ 100$$

Before the following analysis the fish were grinded using a coffee grinder until a homogenous thin sandy aspect was achieved.

### 2.24.2 Protein

Protein and nitrogen contents were analysed using Kjeldahl Method. For protein digestion, 100 mg of each sample was weighted and 10 ml of 98% Sulphuric acid was added in each sample followed by 1 catalyst tablet was added. For protein digestion, the samples were placed in a Kjeldatherm Digestion Block and left for 15 min at 105°C, followed by 60 min at 225°C, and 45 min at 380°C. After samples were cooled they were placed in a Vapodest Destilation unit for distillation and titration. Protein quantifications were calculated by Vapodest Manager software using the following equation:

$$\%\ \mathrm{N} = \frac{(\text{sample titrant} - \text{Blank titrant})\text{x Acid Normality x MW Nitrogen}}{\text{Sample weigth}}$$

Where N = nitrogen, and titrations was calculated in ml. For protein percentage the equation below was used considering conversion factor = 6.25.

$$\%\ \text{Protein} = \%\ \text{Nitrogen x Conversion factor}$$

### 2.24.3 Ash

For total inorganic material or mineral content, an amount of 500 mg of each sample was weighted in a porcelain crucibles and placed in a muffle furnace at 550°C for 8 hours until a light grey ash resulted. The calculations for ash were performed accordingly the following equation:

$$\%\ \mathrm{Ash} = \ 100\ \mathrm{x}\frac{(\text{weight of crucible} + \text{residue}) - \text{weigth of crucible (g)}}{\text{Sample weigth}}$$

### 2.24.4 Lipids

Lipid content was determined using rapid soxhlet extraction. Two grammes of each sample were weighed into cellulose thimbles and put into beakers. After that, 140 ml of solvent (40-60 Petroleum Ether) was added in each beaker and then placed on Soxtherm® unit. Fat percentage was calculated by the following equation:

$$\% \text{ Fat} = 100 \text{ x } \frac{\text{Final weigth of beaker} - \text{Initial weigth of beaker}}{\text{Initial weigth of sample}}$$

**2.25 Statistical analysis**

Data were tested for normality and the Bartlett test was used to verify homogeneity of variance, prior to Tukey analysis for difference of means. Data that did not present homogeneity of variance were submitted to non-parametric *Kruskal-Wallis test.*

High-throughput sequencing data was analyzed using Primer V6.0 software (PRIMER-E Ltd., Ivybridge, UK) using PERMANOVA + V1.0.8 tool (Segata et al., 2011). The permutation analysis were performed with 999 permutations to the weighted and unweighted UniFrac distance matrix from Beta diversity analysis in QIIME within significance at $p<0.05$. The similarities between the microbiota compositions of the intestinal samples from the two fish sizes investigated were compared using weighted principal coordinate analysis (PCoA) and unweighted pair group method with arithmetic (UPGMA). Liner discriminant analysis (LDA) effect size (LEfSe) was used to verify significant differences in OTUs among treatments using the online interface available at http://huttenhower.sph.harvard.edu/galaxy. LEfSe analysis was determined using Alpha value of 0.01 for both Kruskal-Wallis test and pairwise Wilcoxon test; and threshold on the logarithmic LDA score was kept as default (2.0) as well as the strategy for multi-class analysis (all-against-all) (Segata et al., 2011).

# Chapter 3a:
# Characterization of microbiota in *Arapaima gigas* intestine and isolation of potential probiotic bacteria.

# Chapter 3a: Characterization of microbiota in *Arapaima gigas* intestine and isolation of potential probiotic bacteria

## 3a.1 Introduction

During recent decades, Brazilian aquaculture companies have been investing in the production and research of native fish for exportation. Among the native species that have been considered, the pirarucu fish (*Arapaima gigas*) has high potential. The obligate air-breather is endemic to the Amazon basin, it is one of the largest freshwater fish in the world (Brauner et al., 2004). Pirarucu can grow from 19 g to 2560 g in 10 months in a recirculating warm water culture system and studies have attested the huge growth potential of pirarucu either in ponds, cages or recirculation systems (Saint-Paul 1986; Sebrae 2010). The production of this specie has increased more than 4-fold from 2012 to 2014 (FAO 2017). Due to the increase in production, there is increased concern about management and high stocking densities that could lead to disease outbreaks during production and to a use of antibiotics. Thus, the use of environmentally friendly approaches such as feed additives, vaccines and probiotic bacteria to support pirarucu production is considered a high priority.

The use of lactic acid bacteria (LAB) as probiotics is well known, a great deal of research has demonstrated their positive effects on fish health (Dimitroglou et al., 2011; Gatesoupe, 1999; Harikrishnan et al., 2010a; Merrifield et al., 2010; Verschuere et al., 2000). However, the use of probiotics at commercial levels requires comprehensive evaluations of the potential benefits in the fish, which involves both *in vitro* and *in vivo* assessments on new fish species.

One important characteristic to be evaluated for a potential probiotic strain is safety. Among many regulations about probiotic safety, in aquaculture, the use of autochthonous and non-antibiotic resistance strains should be further studied. According to EFSA (European Food Safety Authority, 2012) report, “the selection of micro-organisms for use as feed

additives should be oriented towards the least resistant organism whenever possible", attesting one of the key criterions of potential probiotics selection (Munoz-Atienza et al., 2013). A bacterial antibiotic resistance can be manifested through intrinsic, natural resistance, or acquired resistance. The intrinsic resistance is not horizontally transferable, however, the acquired resistance can occur from mutations or by the acquisition of genes into their genome. These genetic changes lead to spread out the resistance within microbial communities by conjugative plasmids, and mobile elements, allowing the mutated DNA transference through different genus and species (Mathur and Singh, 2005). Therefore, the routine use of antibiotics in fish farms in many parts of the world is leading to the development of antibiotic resistant bacteria. Consequently, the efficacy of antibiotics for treating a number of bacterial diseases are becoming diminished (Defoirdt et al., 2011). The use of prophylactic measures such as probiotics has been studied in recent years to reduce the prophylactic use of antibiotics in aquaculture.

Although there has been some success with probiotics derived from, or designed for, terrestrial animals, a promising strategy is to isolate potentially probiotic LAB from fish (Nayak, 2010). Autochthonous (strains isolated from the fish host) LAB strains are more likely to display the characteristics and properties required to colonize the intestine (Sun et al., 2013). The use of autochthonous LAB strains has been demonstrated to modulate the gut microbiome of fish species, often resulting in positive results for fish immunity and disease resistance (Chi et al. 2014; He et al. 2013; Hjelm et al. 2004; Mouriño et al. 2015; Merrifield and Ringø 2014).

Although often overlooked, assessing the intestine microbiota communities in parallel to the isolation of potential probiotic bacteria is important to understand the relationship of the isolates in relation to the total microbiome, and to identify which members of the gut microbiome should be considered targets for modulation by the potential probiont.

The use of high-throughput sequencing analysis offers a high resolution approach to analyze the composition and diversity of intestinal bacterial communities in fish (Ju and Zhang 2015).

Research on the composition of pirarucu intestinal microbiota is scarce and little is known about potentially probiotic bacteria of this fish. Thus, the aim of this investigation was to isolate and identify potential probiotic bacteria for pirarucu (*A. gigas*) and to assess the *normal* intestinal microbiota of this fish.

## 3a.2 Material and methods

### 3a.2.1 Microbiota analysis

Eight *A. gigas* (five juvenile fish of 8 cm and three adult fish of 150 cm) from semi-intensive culture system pounds from the farm *Mar e Terra Ind. Com. de pescados S/A* located in Rondonia state in Brazil were anesthetized with 0.01% Eugenol (Vetec/Sigma-Aldrich), and euthanized by destruction of the brain. The intestines were aseptically excised and the digesta (intestinal content) and mucosa (intestinal tissue) were sampled. Mucosa samples were washed with sterile phosphate-buffered saline (PBS Oxoid, Basingstoke, UK. All intestinal samples were stored in 98% sterile molecular grade ethanol, centrifuged (17.000g for 5min) prior to the extraction of DNA from 100 mg samples, as described elsewhere (Falcinelli et al. 2015).

To perform high-throughput sequencing analysis the extracted DNA from all samples were used as template for PCRs as described in session 2.11 from Chapter 2.

### 3a.2.2 Probiotic Screening

#### Isolation of bacterial strains

Twenty three *A. gigas* in three different life stages: three fish from post-hatch stage (1.5 cm); nine hatchery fish (8 cm); and eleven adult fish (150 cm) were euthanized and sampled as described previously. The intestines were aseptically excised and rinsed with sterile saline solution (0.65% NaCl, Synth Sao Paulo). The tissue was inoculated in de Man,

Rogosa and Sharpe (MRS, Difco Sao Paulo) broth and incubated overnight at 35 º C (Mourino et al., 2012).

Five *A. gigas* of approximately 8 cm length, from the *Mar e Terra Ind. Com. de pescados S/A* were sampled during a previous, isolated, disease outbreak period. The fish were anesthetized with 0.01% eugenol (Vetec/Sigma-Aldrich) and euthanized. Then, liver, kidney and heart were excised and homogenised in sterile mortar and pestle with 0.65% NaCl solution at the ratio of 1:1.

For further information on isolation of bacterial strains see session 2.3 and 2.4 from Chapter 3.

**16S rRNA sequencing**

The LAB bacteria were grown overnight in MRS broth at 37 ˚C and pathogenic strains in BHI broth at 30˚C, centrifuged at 4,000 g for 10 min and re-suspended in TE buffer. DNA was extracted from the pellets using the CTAB method, adapted from the Joint Genomic Institute (JGI) protocol (William and Copeland 2004). The PCR procedures and identification of the isolates are explained in detail in session 2.6 from Chapter 2.

**Antibiotic resistance**

A number of genes conferring resistance to erythromycin (*erm*(A), *erm*(B), *erm*(C)), tetracycline (*tet*(M), *tet*(L), *tet*(W)) and chloramphenicol (*cat*) were assessed. Antibiotic minimum inhibitory concentration (MIC) for LAB was also assessed using VetMIC$^{TM}$ Lact-1 microplates (National Veterinary Institute, Uppsala, Sweden). The antibiotics included in the microplate were gentamycin, kanamycin, streptomycin, neomycin and tetracycline, erythromycin, clindamycin and chloramphenicol. Detailed methodology is clarified in session 2.12 from Chapter 2.

***In vitro* antagonism of fish pathogenic bacteria by potentially probiotic strains.**

The antagonistic properties of the LAB isolates, against the pathogenic strains, was evaluated in triplicate, using the agar disk diffusion antagonism technique as described by Vieira et al. (2007). See session 2.13 from Chapter 2 for detailed methodology.

**Haemolytic assay**

Fresh colonies for all LAB stains were plated on triplicate blood agar plates. Blood agar plates were examined for signs of β-haemolysis (clear zones around colonies), α-haemolysis (green-hued zones around colonies) or γ-haemolysis (no zones around colonies).

**Viability of LAB in diets**

The non-haemolitic LAB were evaluated for viability in fish diets. Complete methodology is referenced in session 2.15 from Chapter 2.

**Statistical analysis**

Data for antagonism, phylum relative abundance and LAB viability in diets were tested for normality and the Bartlett test was used to verify homogeneity of variance, prior to Tukey analysis for difference of means using SPSS software. For high-throughput sequencing statistic see session 2.24 from Chapter 2.

## 3a.3 Results

***Arapaima gigas* intestinal microbiome**

High-throughput sequencing analysis of bacterial 16S rRNA V1-V2 regions from the Ion Torrent® PGM yielded a total of 5,325,282 raw sequences. After removal of low quality reads, filtering 0.005% and removal of *Streptophyta* (69,104 reads; which was considered a contaminant from chloroplasts in feed materials) (Gajardo et al. 2016) a total 1,246,730 reads were used for downstream analysis. These comprised 600,366 sequences were from juvenile mucosa samples, 597,010 from juvenile digesta samples, 28,262 from adult mucosa samples and 21,092 sequences from adult digesta samples. The Good's

coverage estimators for all sample types were >0.995 indicating that good coverage was achieved.

In terms of sequence distribution, Fusobacteria, Proteobacteria, Firmicutes and Bacteroides constituted the majority of phylum composition (Figure 3a.1). ANOVA analysis reveals difference in Proteobacteria abundance when compared juvenile and adult fish. However, Firmucutes phylum was more abundant in juvenile fish digesta when compared with adult fish (Table 3a.1).

The relative abundance of reads assigned to the genera level (Figure 3a.2) in adult fish was greatest for *Bradyrhizobium* (accounting for 35% and 30% of reads in the digesta and mucosa, respectively) followed by *Cetobacterium* (29% for both intestinal regions). The relative abundance of *Cetobacterium* sequence reads was also high in mucosa (11%) and digesta (41%) of juvenile fish, followed by reads assigned to the Clostridiales order (6%) in digesta and *Lactococcus* (9%) in mucosa.

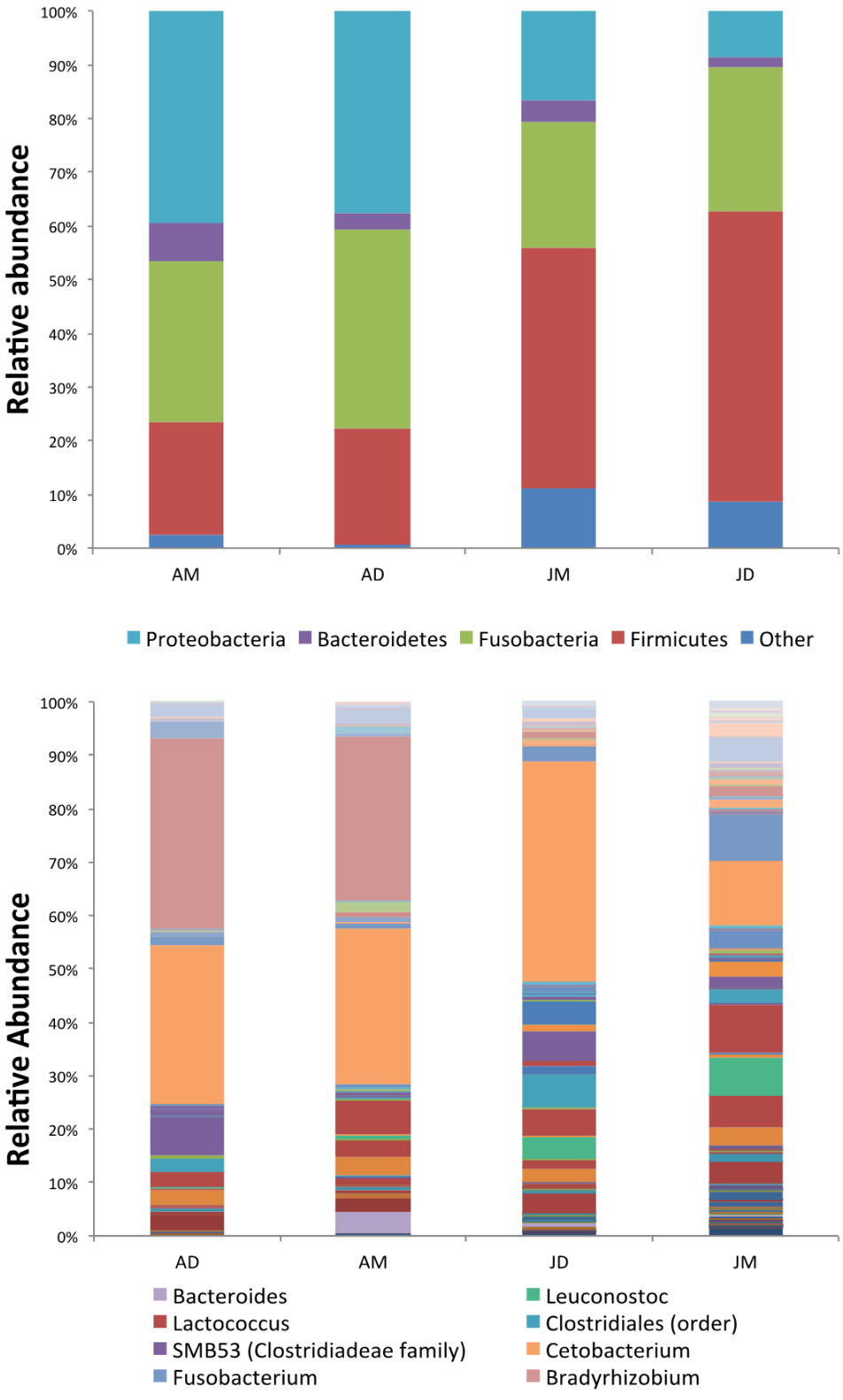



Figure 3a.1: Taxonomic composition of the average of relative abundance (%) – phylum level (A) and genus level (B). The plots represent the four most abundant representatives in each category juvenile fish mucosa (JM), juvenile fish digesta (JD), adult fish mucosa (AM) and adult fish digesta (AD)

Table 3a.1: Relative abundance (mean % ± standard deviation) at phylum level of intestinal microbiota of pirarucu (*A. gigas*) juvenile fish mucosa (JM), juvenile fish digesta (JD), adult fish mucosa (AM) and adult fish digesta (AD)

| Treatment | Other | Firmicutes | Fusobacteria | Bacteroidetes | Proteobacteria |
|---|---|---|---|---|---|
| JM | 10.99±6.92$^{b}$ | 44.77±21.17$^{ab}$ | 23.57±28.28 | 4.01±5.48$^{ab}$ | 16.65±10.39$^{a}$ |
| JD | 8.81±8.57$^{ab}$ | 53.76±27.28$^{b}$ | 27.07±27.99 | 1.73±1.01$^{a}$ | 8.63±8.48$^{a}$ |
| AM | 2.51±2.15$^{ab}$ | 21.05±6.01$^{a}$ | 29.85±13.1 | 7.14±4.07$^{b}$ | 39.45±10.8$^{b}$ |
| AD | 0.58±0.46$^{a}$ | 21.52±13.76$^{a}$ | 37.11±11.44 | 3.17±2.59$^{ab}$ | 37.62±18.39$^{b}$ |

*Different superscripts indicate a significant difference ($p<0.05$)

Alpha diversity measures revealed statistical differences between fish life stage (Table 3a.2). It is clear that juvenile fish gut microbiota is differed from the microbiota of adult fish (Figure 3a.2). The number of OTUs (in both mucosa and digesta samples) in juvenile fish was significantly higher than in adult fish. When analysing separately the groups in fish tissue (mucosa x digesta) and life stage (juvenile x adult), the PCoA plot analysis revealed a general spatial separation of communities by life stage and a higher similarity was observed within adult fish microbiota samples when compared with juvenile fish (Figure 3a.3c and 3a.3d). PERMANOVA unweighted results confirm PCoA findings and shows that *A. gigas* microbiota (genus level) differs between life stage, however, no difference was recorded within mucosa and digesta in juvenile fish (Table 3). On the other hand, weighted results of PERMANOVA shows that mucosa and digesta microbiota diversity don't differ within juvenile and adult in *A. gigas*' gut as well as juvenile and adult digesta.

Table 3a.2: Alpha parameters results (Chao1 index, Observed Species, and PD Whole Tree) of intestinal microbiota composition of pirarucu (*A. gigas*) juvenile fish mucosa (JM), juvenile fish digesta (JD), adult fish mucosa (AM) and adult fish digesta (AD)

| Treatments | Chao1 index | Observed Species | Phylogenetic diversity (PD) |
|---|---|---|---|
| JM | $357.70 \pm 78.99^{b}$ | $239.03 \pm 65.33^{b}$ | $12.00 \pm 2.86^{b}$ |
| JD | $330.09 \pm 87.02^{b}$ | $261.34 \pm 53.30^{b}$ | $10.11 \pm 3.05^{b}$ |
| AM | $194.98 \pm 17.33^{a}$ | $145.43 \pm 12.64^{a}$ | $6.08 \pm 0.60^{a}$ |
| AD | $174.97 \pm 25.03^{a}$ | $122.78 \pm 15.81^{a}$ | $5.60 \pm 0.93^{a}$ |

*Different superscripts indicate a significant difference ($p<0.05$).

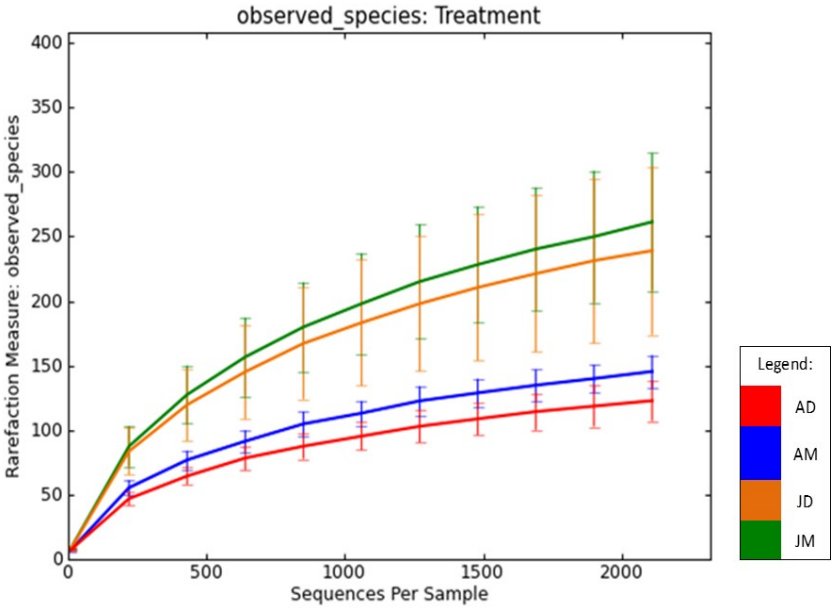



Figure 3a.2: Rarefaction curve for observed species (OTUs) for juvenile fish mucosa (JM) and digesta (JD); and adult fish mucosa (AM) and digesta (AD). Showing difference between sizes

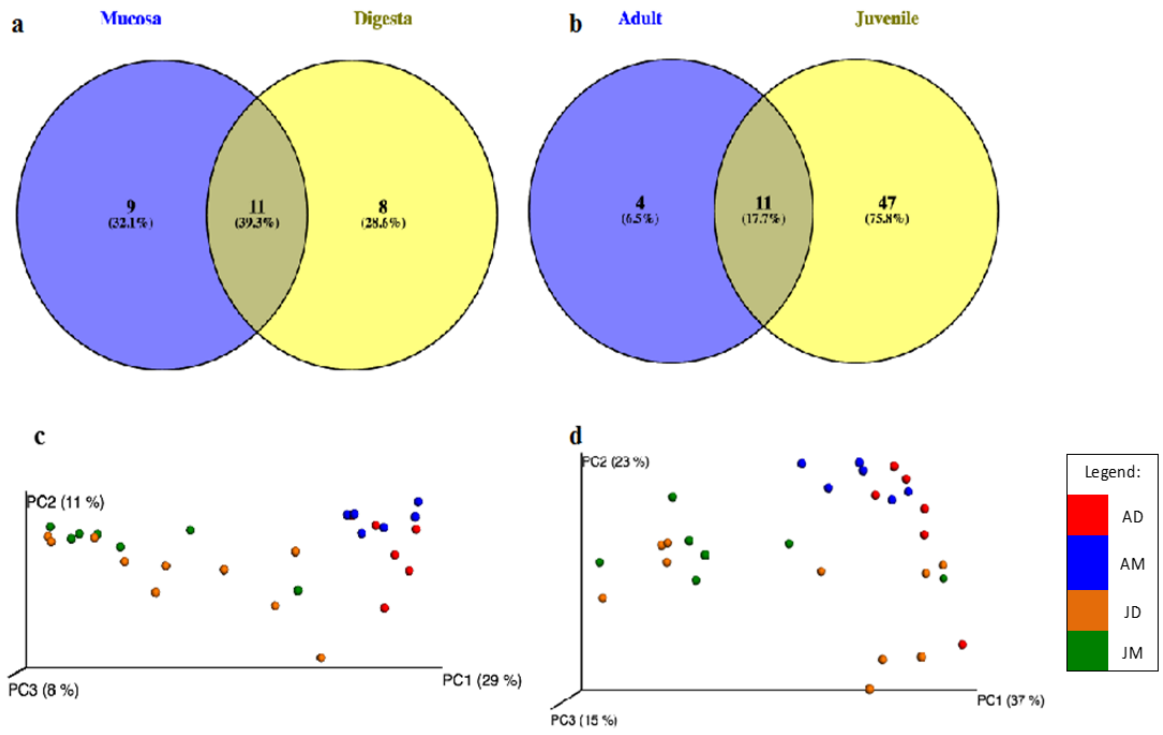



Figure 3a.3: Venn diagram for unique and shared OTUs (species) for fish tissue (a) and life stage (b) showing 80% of samples in each compartment; and PCoA or unweight (c) and weighted UniFrac (d) showing clustering of compartments for adult fish digesta (AD), adult fish mucosa (AM), juvenile fish digesta (JD) and juvenile fish mucosa (JM)

The core microbiota was calculated based on the presence of respective OTUs (at species level) in 80% of samples. A total of 11 OTUs: *Cetobacterium somerae; Lactococcus garvieae; Lactococcus* sp.*; Staphylococcus epidermidis; Bradyrhizobium elkanii; Enterobacteriaceae* (family)*; Trabulsiella* sp.*; Bradyrhizobium* sp.*; Erysipelotrichaceae* (family)*; Propionibacterium acnes; Brevibacillus reuszeri.* comprised the core microbiota between juvenile and adult *A. gigas* (Figure 3a.3a). Analysis between mucosa and digesta revealed a core microbiota comprising 11 OTUs: *Cetobacterium somerae; Staphylococcus epidermidis; Enterococcus* sp.*; Lactococcus garvieae; Trabulsiella* sp.*; Bradyrhizobium elkanii; Bradyrhizobium* sp.*; Propionibacterium acnes; Afipia felis; Brevibacillus reuszeri; Rhizobiales* (order) (Figure 3a.3b).

Table 3a.3: PERMANOVA results of unweight and weighted UniFrac showing difference between categories of intestinal microbiota composition of Pirarucu (*A. gigas*): juvenile fish mucosa (JM), juvenile fish digesta (JD), adult fish mucosa (AM) and adult fish digesta (AD).

| | | **PERMANOVA** | **Pair-wise test** | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | JM x JD | JM x AD | JM x AM | JD x AD | JD x AM | AD x AM |
| Unweighted | p-value | 0.001 | 0.705 | 0.002 | 0.002 | 0.001 | 0.001 | 0.005 |
| | Pseudo-F/ t-value | 3.3951 | 0.889 | 2.147 | 2.199 | 2.052 | 2.139 | 1.366 |
| Weighted | p-value | 0.002 | 0.742 | 0.006 | 0.001 | 0.012 | 0.002 | 0.077 |
| | Pseudo-F/ t-value | 3.111 | 0.703 | 2.183 | 2.094 | 1.901 | 2.105 | 1.597 |

**Identification of LAB isolates**

The molecular identification of the 23 LAB isolated from the fish gut revealed six different species including: four *Enterococcus faecalis* strains, seven *Lactococcus lactis* subsp. *lactis* strains, five *Weissella paramesenteroides* strains, three *Staphylococcus* sp. strains, four *Lactococcus garvieae* strains and one *Enterococcus faecium* strain. The six isolates from diseased *A. gigas* were identified as: two *Citrobacter freundii* strains, two *Pseudomonas* sp. strains, one *Enterobacter* sp. strain, and one *Pseudomonas stutzeri* strain (Table 3a.4).

Table 3a.4: Molecular identification of lactic acid bacteria (LAB) strains and potential pathogenic strains using 16S rRNA gene and BLAST at GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

| **LAB** | | | | | |
|---|---|---|---|---|---|
| **Fish size (cm)** | **Molecular identification** | **Strain label** | **Fish size (cm)** | **Molecular identification** | **Strain label** |
| 1.5 | *S. epidermidis* | G23 | 150 | *Staphylococcus* sp. | G27 |
| | *L. lactis* subsp. *lactis* | G24 | | *W. paramesenteroides* | G28 |
| | *W. paramesenteroides* | G25 | | *W. paramesenteroides* | G29 |
| 8 | *E. faecalis* | PCA | | *S. epidermidis* | G31 |
| | *E. faecalis* | PCB | | *E. faecalis* | G32 |
| | *L. lactis* subsp. *lactis* | G1 | | *L. garvieae* | G33 |
| | *L. lactis* subsp. *lactis* | G4 | | *L. garvieae* | G34 |
| | *L. lactis* subsp. *lactis* | G5 | | *W. paramesenteroides* | G35 |
| | *L. lactis* subsp. *lactis* | G7 | | *L. garvieae* | G36 |
| | *L. lactis* subsp. *lactis* | G9 | | *L. garvieae* | G38 |
| | *E. faecalis* | G12 | | *E. faecium* | G40 |
| | *W. paramesenteroides* | G16 | | | |
| **Pathogenic** | | | | | |

| Fish size (cm) | Molecular identification | Strain label | Fish size (cm) | Molecular identification | Strain label |
|---|---|---|---|---|---|
| 8 | *C. freundii* | PP1 | 8 | *Enterobacter.* sp. | PP4 |
| | *Pseudomonas* sp. | PP2 | | *P. stutzeri* | PP5 |
| | *C. freundii* | PP3 | | *Pseudomonas* sp. | PP6 |

**Antibiotic resistance**

The presence of ARGs was detected after PCR in bacterial strains G4 (*L. lactis* subsp. *lactis*), G16 (*W. paramesenteroides*), G24 (*L. lactis* subsp. *lactis*), G28 (*W. paramesenteroides)*, G32 (*E. faecalis*), G34 (*L. garvieae*), G35 (*W. paramesenteroides*), G36 (*L. garvieae*), one (*L. garvieae*). In general, only three strains isolated from juvenile fish (from 12 total strains) presented positive results for resistance while six strains isolated from adult fish (from 11 total strains) (Table 3a.5). From all the isolates, only nine strains: four *L. lactis* subsp. *Lactis*, one *S. epidermidis*, two *W. paramesenteroides,* one *Staphylococcus* sp., and one *E. faecium* did not present antibiotic resistance after the MIC analysis from all the antibiotics tested (Table 3a.6).

Table 3a.5: Presence of antibiotic resistance genes in bacteria isolated from *A. gigas* gut.

| Label | Strains | Antibiotic resistant genes | | | | | |
|---|---|---|---|---|---|---|---|
| | | ***erm*(A)** | ***erm*(B)** | ***erm*(C)** | ***tet*(M)** | ***tet*(L)** | ***cat*** |
| PCA | *E. faecalis* | - | - | - | - | - | - |
| PCB | *E. faecalis* | - | - | - | - | - | - |
| G1 | *L. lactis* subsp. *lactis* | - | - | - | - | - | - |
| G4 | *L. lactis* subsp. *lactis* | - | + | - | - | - | - |
| G5 | *L. lactis* subsp. *lactis* | - | - | - | - | - | - |
| G7 | *L. lactis* subsp. *lactis* | - | - | - | - | - | - |
| G9 | *L. lactis* subsp. *lactis* | - | - | - | - | - | - |
| G12 | *E. faecalis* | - | - | - | - | - | - |
| G16 | *W. paramesenteroides* | - | - | + | - | - | - |
| G23 | *S. epidermidis* | - | - | - | - | - | - |
| G24 | *L. lactis subsp. lactis* | - | + | - | - | - | + |
| G25 | *W. paramesenteroides* | - | - | - | - | - | - |
| G27 | *Staphylococcus* sp. | - | - | - | - | - | - |
| G28 | *W. paramesenteroides* | - | + | + | - | - | + |
| G29 | *W. paramesenteroides* | - | - | - | - | - | - |
| G31 | *S. epidermidis* | - | - | - | - | - | - |
| G32 | *E. faecalis* | - | - | + | - | - | - |
| G33 | *L. garvieae* | - | - | - | - | - | - |
| G34 | *L. garvieae* | + | + | - | - | - | + |
| G35 | *W. paramesenteroides* | - | - | + | - | - | - |
| G36 | *L. garvieae* | - | - | - | - | - | + |
| G38 | *L. garvieae* | + | + | - | - | - | - |
| G40 | *E. faecium* | - | - | - | - | - | - |

Table 3a.6: Minimal Inhibitory Concentration (MIC) of antibiotics against 23 isolated lactic acid bacteria (LAB) from *A. gigas* intestine

| Antibiotic | Strain | Number of tested strain / Number of stains above EFSA limit | EFSA limit (mg/L) |
|---|---|---|---|
| Gentamicin | *L. lactis* subsp. *lactis* | 6/0 | 4 |
| | *Weissella* sp. | 5/1 | 0.25 |
| | *E. faecium* or *faecalis* | 5/4 | 4 |
| | *Staphylococcus* sp. | 3/0 | 0.25 |
| | *L. garvieae* | 4/2 | 2 |
| Kanamycin | *L. lactis* subsp. *lactis* | 6/0 | 64 |
| | *Weissella* sp. | 5/1 | 16 |
| | *E. faecium* or *faecalis* | 5/0 | 1024 |
| | *Staphylococcus* sp. | 3/0 | n.a |
| | *L. garvieae* | 4/1 | 16 |
| Streptomycin | *L. lactis* subsp. *lactis* | 6/0 | 64 |
| | *Weissella* sp. | 5/0 | 16 |
| | *E. faecium* or *faecalis* | 5/0 | n.a |
| | *Staphylococcus* sp. | 3/0 | n.a |
| | *L. garvieae* | 4/1 | 8 |
| Neomycin | *L. lactis* subsp. *lactis* | 6/0 | n.a |
| | *Weissella* sp. | 5/0 | n.a |
| | *E. faecium* or *faecalis* | 5/0 | n.a |
| | *Staphylococcus* sp. | 3/0 | n.a |
| | *L. garvieae* | 4/0 | n.a |
| Tetracycline | *L. lactis* subsp. *lactis* | 6/1 | 4 |
| | *Weissella* sp. | 5/3 | 2 |
| | *E. faecium* or *faecalis* | 5/0 | 4 |
| | *Staphylococcus* sp. | 3/0 | 1 |
| | *L. garvieae* | 4/3 | 2 |
| Erythromycin | *L. lactis* subsp. *lactis* | 6/1 | 2 |
| | *Weissella* sp. | 5/2 | 0.5 |
| | *E. faecium* or *faecalis* | 5/0 | 4 |
| | *Staphylococcus* sp. | 3/1 | 1 |
| | *L. garvieae* | 4/1 | 0.5 |
| Clindamycin | *L. lactis* subsp. *lactis* | 6/0 | 4 |
| | *Weissella* sp. | 5/1 | 0.25 |
| | *E. faecium* or *faecalis* | 5/4 | 4 |
| | *Staphylococcus* sp. | 3/0 | 0.25 |
| | *L. garvieae* | 4/2 | 2 |
| Chloramphenicol | *L. lactis* subsp. *lactis* | 6/0 | 8 |
| | *Weissella* sp. | 5/1 | 2 |
| | *E. faecium* or *faecalis* | 5/0 | 16/32[c] |
| | *Staphylococcus* sp. | 3/0 | 16 |
| | *L. garvieae* | 4/2 | 2 |

[a] MICs determined by a VetMIC test. The antibiotic dilution ranges were: 0.03-16 $mg.L^{-1}$ (clindamycin), 0.5-256 $mg.L^{-1}$ (gentamicin, streptomycin and neomycin), 2-1024 $mg.L^{-1}$ (kanamycin), 0.016-8 $mg.L^{-1}$ (erythromycin), 0.12-64 $mg.L^{-1}$ (tetracycline, chloramphenicol).
[b] LAB with MICs higher than the EFSA breakpoints are considered as resistant strains (EFSA 2012). [c] Break point 16 to *E. faecium* and 32 to *E. faecalis.*
* n.a., not available.

***In vitro* antagonism**

The isolates G1, G4, G5 and G9 (*L. lactis* subsp. *lactis*), G25 (*W. paramesenteroides*), and G40 (*E. faecium*) were selected for this assay based on lack of historical records of disease in fish by these species as well as the lack of ARGs. The halos were accepted as positive antagonism if the average was > 0.8 cm (Table 3a.7).

The largest mean halo diameter against all pathogens was observed on the G5 *L. lactis* subsp. *lactis* (1.45 ± 0.38 cm) followed by G25 *W. paramesenteroides* (1.45 ± 0.27 cm) when compared with the other LAB isolated. The strains G4 (*L. lactis* subsp. *lactis*) G25 (*W. paramesenteroides*) and G40 (*E. faecium*) had positive antagonism against all the pathogenic bacteria tested. While the strains G5, G4 (*L. lactis* subsp. *lactis*), G25 (*W. paramesenteroides*) and G40 (*E. faecium*) had positive antagonism against the bacteria isolated from *A. gigas*.

**Haemolytic assay**

The LAB isolates G1 (*L. lactis* subsp. *lactis*), G5 (*L. lactis* subsp. *lactis*) and G40 (*E. faecium*) did not present haemolysis on blood agar plates. However the bacteria G4 (*L. lactis* subsp. *lactis*) G9, (*L. lactis* subsp. *lactis*) and G25 (*W. paramesenteroides*) presented α haemolysis and consequently discarded as potential probiotic.

**Viability of LAB in diets**

During the inoculation process and drying temperature at 50 °C, both LAB strains G5 (*L. lactis* subsp. *lactis*) and G40 (*E. faecium*) were still viable three weeks after the inoculation in fish diet (Table 3a.8). However, the strain G40 had a further decrease of concentration at the third week (15%) when compared with the initial inoculation.

Table 3a.7: Antagonism halos (average in cm ± standard deviation) of LAB G1, G4, G5 and G9 (*L. lactis* subsp. *lactis*), G25 (*W. paramesenteroides*), and G40 (*E. faecium*) against possible pathogenic bacteria: PP1 (*Citrobacter freundii*), PP2 (*Pseudomonas* sp.), PP3 (*C. freundii*), PP4 (*Enterobacter* sp.), PP5 (*P. stutzeri*), PP6 (*Pseudomonas* sp.); and standard fresh water pathogens: ML (*Micrococcus luteus*), AH1 (*Aeromonas hydrophila* ATCC 7966), SA (*Staphylococcus agalactiae*) and AH2 (*A. hydrophila* DRM CPQBA 228-08)

| Strains | PP1 | PP2 | PP3 | PP4 | PP5 | PP6 | ML | AH1 | SA | AH2 |
|---|---|---|---|---|---|---|---|---|---|---|
| **G5** | 1.4±0.1 | 2.0±0.2 | 1.5±0.2 | 1.4±0.2 | 2.0±0.2 | 1.7±0.3 | 1.4±0.1 | na | 1.1±0.3 | 1.3±0.4 |
| **G25** | 1.7±0.2 | 1.4±0.2 | 1.6±0.3 | 1.2±0.2 | 1.2±0.2 | 1.6±0.1 | 1.7±0.4 | 1.7±0.1 | 1.2±0.1 | 1.3±0.2 |
| **G40** | 1.7±0.2 | 1.6±0.2 | 1.7±0.1 | 0.9±0.1 | 1.6±0.4 | 1.2±0.2 | 0.8±0.0 | 1.4±0.1 | 1.1±0.1 | 1.3±0.2 |
| **G4** | 1.4±0.3 | 1.1±0.3 | 1.3±0.1 | 1.2±0.2 | 1.2±0.0 | 1.4±0.1 | 1.2±0.2 | 1.3±0.1 | 1.4±0.4 | 1.1±0.2 |
| **G9** | 1.4±0.1 | 1.2±0.3 | 1.3±0.2 | na | 1.0±0.2 | 1.0±0.1 | 1.1±0.2 | na | na | na |
| **G1** | 1.1±0.3 | 1.0±0.2 | 1.3±0.3 | na | 0.9±0.1 | 0.9±0.1 | 1.1±0.2 | 1.1±0.2 | 1.3±0.1 | 1.1±0.3 |

*na= no antagonism observed (no halo)

**Different superscripts indicate a significant difference ($p<0.05$)

Table 3a.8: Average and standard deviation of LAB viability (%) in diets after inoculation of $1x10^9$ ($CFU.ml^{-1}$)

| Strain | Innoculation(1) | Week 1 | Week 2 | Week 3 |
|---|---|---|---|---|
| **G5** | $100\pm0.00^{b}$ | $93.11\pm1.83^{a}$ | $97.12\pm2.54^{b}$ | $96.91\pm0.46^{ab}$ |
| **G40** | $100\pm0.00^{b}$ | $98.4\pm2.19^{b}$ | $99.19\pm3.97^{b}$ | $84.81\pm4.47^{a}$ |

*G5 (*L. lactis* subsp. *lactis*) and G40 (*E. faecium*)
(1) Counts after drying process
**Different superscripts indicate a significant difference ($p<0.05$)

## 3a.4 Discussion

Assessing the core bacteria community in *A. gigas* can help to provide fundamental information about the *normal* abundance of the potential probiotic candidates identified in the current study and also identifies which potential pathogens inhabit the gut of pirarucu. No previous work has described the intestinal bacteria present in intestine under normal conditions of fish farming in pounds for this species of fish. This study has attested that important differences were found in the gut microbiota of adult and juvenile fish. *In vitro* results have suggested that two autochthonous strains from *A. gigas* have potential probiotic effect.

*A. gigas microbiome*

After performing bioinformatics analysis using QIIME, data diversity analysis output, which includes Alpha-diversity analysis, was used to compare species richness is by rarefaction curve within one sample (Ju and Zhang 2015). In this study, Alpha diversity analysis suggests that juvenile fish mucosa was richer in number of species compared with adult fish digesta. This result was also observed in small Atlantic cod larvae (*Gadus morhua*) that showed more diverse intestine microbiota diversity and evenness when compared with large larvae (Forberg et al., 2016). It seems that juvenile fish microbiota are more likely to interact with the environment or different diets than adults. Host factors are stronger in adults so the selection pressure

is higher decreasing number of bacteria that could colonize the intestine (Forberg et al., 2016).

Anaerobic bacteria from *Cetobacterium* genus, in fresh water fish, are related with vitamin B12 production and peptides fermentation (Tsuchiya et al., 2008). Similar to the present study, *Cetobacterium* was also the most abundant genus present in intestine of freshwater species such as carp (*Ctenopharyngodon idellus, Carassius cuvieri and Hypophthalmichthys nobilis*) (Li et al., 2015), channel catfish (*Ictalurus punctatus*), largemouth bass (*Micropterus salmoides*) and bluegill (*Lepomis macrochirus*) (Larsen et al., 2014). The most abundant phyla present in the pirarucu intestine were Proteobacteria, Fusobacteria and Firmicutes in both lfe stages and all tissues, which were also reported to be highly abundant in the gut of other freshwater fish such as brown trout (*Salmo trutta*), channel catfish (*Ictalurus punctatus*), largemouth bass (*Micropterus salmoides*) and bluegill (*Lepomis macrochirus*) (Al-Hisnawi et al., 2014; Larsen et al., 2014).

The list of bacterial species shared by all individuals of a given host species is known as the core microbiota (Li et al., 2015). Core fish microbiota can change according fish life stage, diets or stocking densities (Wong et al., 2013). The low percentage (17.7%) of core microbotia regarding different life stage (juvenile x adult) suggests that this microbiota is specific for life stage and only few species/OTUs are able to colonize the intestine of different conditions such as age of *A. gigas*. Theseresults are not observed between digesta and mucosa samples where the percentage of core microbiota was higher (39.3%). These results suggest that the size of the fish is the main driver of the differences between the microbiota more than the tissue. Accordingly, the bacterial community associated with life stage displayed

higher similarity (PCoA Plot) than the ones associated with fish tissue suggesting that the adult fish community is more stable than the juvenile fish community samples.

*LAB isolates*

Between all the identified isolates form Pirarucu intestine, the species *Lactococcus garvieae* and *Staphylococcus epidermidis* are known as being pathogens in rainbow trout (*Oncorhynchus mykiss*) (Bastardo et al., 2012) as well as in sea bream (*Sparus aurata*) (Austin and Austin 2007). Strains that present a pathogenic background should be avoid to be used as probiotic bacteria (Merrifield et al., 2010), consequently they were discarded from the in vitro test to be used as probiotic.

On the other hand, the strains identified as *Enterococcus*, *Lactococcus* and, *Weissella* have been reported by Newaj-Fyzul et al. (2014) as probiotic genus commonly used in aquaculture. The application of *E. faecium* reduced edwardsiellosis mortalities in European eel (*Anguilla anguilla*) (Chang and Liu 2002). This species was also reported that improve common sole (*Solea solea*) larval growth performance (Avella et al., 2011) as well as improves immune response and increase growth performance in Nile tilapia (Wang et al., 2008). Besides of being reported as fish pathogen (Austin and Austin 2007), the bacteria *E. faecalis* was isolated from gastrointestinal tract of rainbow trout and was also described as LAB with beneficial properties in fish (Merrifield and Ringø 2014). This strain present positive results in rainbow trout promoting growth and immune stimulation (Rodriguez-Estrada et al., 2013). The genera *Lactococcus* and *Enterococcus* are part of Firmicutes phylum, and they were isolated from the juvenile fish intestine samples in the present study. In adult fish data this phylum was not the most abundant, concluding that the use of these strains could be an alternative to increase its abundance in adult fish helping them to fight against diseases during adult stage.

Strains of *Lactococcus lactis* were previously tested as probiotics in olive flounder (*Paralichthys olivaceus*) resulting in an activation of the innate immune system of this species (Kim et al., 2013; Heo et al., 2013). Similar results were reported in brown trout and rainbow trout (Balcazar et al., 2007a; Balcázar et al., 2007b). *Lactococcus lactis* strains have been isolated from several fish gut species such as Atlantic cod (*Gadus morhua*), Patagonian fish (*Odontesthes platensis*), yellow grouper (*Epinephelus awoara*), Amur catfish (*Silurus asotus*), common carp (*Cyprinus carpio*), and grass carp (Lauzon et al., 2014) indicating that this species is a component of the normal gut microbiota of freshwater and marine fish.

To the authors' knowledge, *Weisella paramesenteroides* has not been described in the literature as probiotic species in aquaculture. However, it has been isolated from rainbow trout distal gut contents (Desai et al., 2012). Strains from the same genus, such as *W. cibaria* and *W. confusa,* were reported to be present in gastrointestinal tract of brown trout and Atlantic salmon (Al-Hisnawi et al., 2014; Hovda et al., 2012). *W. cibaria* has been isolated from hybrid surubim (*Pseudoplatystom*a sp.), a native Brazilian fish, and its application as probiotic to this fish species reduced the numbers of pathogenic bacteria and stimulated the presence of LAB in intestinal microbiota (Mourino et al., 2012).

After analysing the *normal* microbiota in pirarucu's intestine, the evaluation of autochthonous isolated from gut microbiota in *A. gigas* as potential probiotic was required. Based on probiotic criteria proposed by Merrifield et al. (2010), the autochthonous strains were submitted to several analyses.

*Antibiotic resistance*

It is well known the antibiotic resistance genes in probiotic bacteria are undesirable from a human and animal production perspective, however, there is a lack of information about the antibiotic resistance of some probiotic strains used in

aquaculture. This study evaluated antibiotic resistance by molecular detection of antibiotic resistance for the main antibiotic used in Brazilian aquaculture. The isolated stains were selected from fish that were already treated with oxytetracycline, which is an antibiotic from tetracycline family; however, no resistance to tetracycline was observed within the isolates. On the other hand, the strains of *Lactococcus lactis* subsp. *lactis* (G4 and G24) present genes for *erm* (B) and *cat* after PCR. Comparing with probiotic bacteria isolated from milk, the strain *Lactococcus lactis* K214 was reported as containing *cat* gene conferring resistance to chloramphenicol and *erm* (B) gene conferring resistance to erythromycin (Ammor et al., 2007). Mathur and Singh (2005) described a presence of a multiple antibiotic resistance plasmid pK214 in *L. lactics*, isolated from soft cheese, which is able to encode streptomycin, tetracycline and chloramphenicol resistance genes. Other studies on commercial probiotics have reported *Weissella* sp. containing the *erm* (B) gene (Sharma et al., 2014), which is in agreeing with the present study.

Besides the acquired resistance of the isolates described previously, the strains can have also intrinsic resistance, or even both characteristics. The strains G16 (*W. paramesenteroides*) and G24 (*L. lactis* subsp. *lactis*) had positive results in both characteristics for *erm*(C) *and erm* (B) respectively. According to Munoz-Atienza et al. (2013), 60% of the non-enterococcal strain *Weissella* sp. presented resistance to antibiotics in VetMic$^{TM}$ analysis. It suggests that acquired resistance may vary between strains from the same genus and not always show resistance., Accordingly with the present study, only half of the strains from genus *Weissella* presented resistance to erythomicin and tetracyclin in VetMic$^{TM}$ analysis..

The transference of resistant genes can occur commonly between gastrointestinal bacteria and pathogenic bacteria, and also between bacteria and host

(Mathur and Singh 2005) depending of the environmental conditions. Therefore, the main reason to choose a probiotic bacterium with no resistance against antibiotics is to avoid the transference of resistance genes to pathogenic bacteria that could inhabit the host.

In the present study, strains G24 (*L. lactis* subsp. *lactis*), G28 (*W. paramesenteroides*), G34 and G38 (*L. garviae*), contained more than one antibiotic resistance gene. According to Hatha et al. (2005), the exchange of resistant plasmids between bacteria spread more easily in aquatic environment and can result in a higher frequency of multiple antibiotic resistant strains (MAR). Therefore, there is a concern about the use of these strains since they may transfer genes to other environmental microorganisms.

Considering the fact that the food chain is the main route of antibiotic resistance bacteria transmission to human population, the aquaculture production companies and research should be involved together to avoid this pathway, by decreasing then, the use bacteria already reported as resistant in aquaculture farms. Though, bacteria that contain antibiotic resistance genes are not suitable as potential probiotics and therefore they were discarded for further analysis in the present study (Merrifield et al. 2010).

*Probiotic properties of LAB isolates from A. gigas intestine*

A wide range of papers have demonstrated the capacity of LAB isolated from fish in inhibiting pathogen such as *Bacillus* sp. isolated from gut of Nile tilapia against *Aeromonas hydrophila*, *Edwardsiella tarda*; *Pseudomonas fluorescens*; and *Pseudomonas putida* (Del'Duca et al., 2013). The antagonistic effect of LAB against pathogens are probably mediated by competition for nutrients and adhesion sites, release of metabolites (organic acids and hydrogen peroxide), and production of bacterocins (Ringø et al. 2010). Other studies using a similar method used in the

present study reported the capacity of *Bacillus amyloliquefaciens* in inhibiting common aquaculture pathogens for example *Edwardsiella tarda*, *Aeromonas hydrophila* and *Vibrio harveyi* presenting antagonism zone (Das et al., 2013). In the present study the isolates from *Arapaima gigas*, G5 *Lactococcus lactis* subsp. *lactis*, G25 *Weissella paramesenteroides* and G40 *Enterococcus faecium*, had positive antagonism against all the isolated pathogens strains from pirarucu and also some other fish pathogens.

In fish production there is concern about the use of a safe probiotic bacteria in fish food (FAO/WHO 2002), because the fish will go into the human food chain and thus the bacteria may also be introduced into this chain. In the present study, the strains G1 and G5 (*L. lactis* subsp. *lactis*), and G40 (*E. faecium*) have shown negative haemolysis (gamma), and given their other positive attributes observed in the present study, they can be considered potential candidates to be used as probiotics in for this species.

In the present study, both probiotic candidates (G5 *Lactococcus lactis* subsp. *lactis* and G40 *Enterococcus faecium*) were able to keep alive during three weeks in diets. Statistic difference reveals that the strain of *L. lactis* subsp. *lactis* had the capacity to keep concentration after a three weeks period while the strain of *E. faecium* decreased its concentration. The ability of a probiont to keep survive in diets is important because feed this is the most common delivery mechanism of probiotics in aquacuture. This is an important probiotic characteristic because the diets are prepared, transported and stored for periods ranging from days to months prior to delivery to the fish. This way the probiotic feeding management could be planned and scheduled in advance by the farmer. Indeed, spray administration of probiotic followed by drying the pellet is a valid method used for on-farm probiotic feed

preparations, and commercial feed mills use top coating using a vacuum method. Similar results reporting the viability of probiotics in fish diets after using spray and dry methods were previously reported (Harikrishnan et al., 2010b; Mouriño et al., 2016) .

It is important to highlight that both final isolates are part of core microbiota of *A. gigas* under this specific farm condition. The strain *L. lactis* subsp. *lactis* is part of core microbiota for juvenile and adult fish; and *E. faecium* was present in both mucosa and digesta samples in at least 80% of the fish analysed in both cases, therefore they are both probiotic candidates.

## 3a.5 Conclusion

Further, this study has demonstrated, for the first time, the presence of LAB strains in the *A. gigas* intestine, and demonstrated their antibiotic resistance and antagonistic behavior against pathogens isolated from the same fish. After determining the *natural* abundance of probiotic LAB candidates the intestinal microbiota of pirarucu at two life stages, future *in vivo* studies are necessary to validate their potential and also to assess the manipulation of the *natural* microbiota after administration of these potential probiotic strains.

# Chapter 3b:
# Lethal dose and pathogenicity characterization of two bacterial strains isolated from pirarucu, *Arapaima gigas*

# Chapter 3b: Lethal dose and pathogenicity characterization of two bacterial strains isolated from pirarucu, *Arapaima gigas*

## 3b.1 Introduction

Due to the fast growth of the aquaculture industry in Brazil there is a concern about the spread of bacterial diseases. In Brazilian aquaculture, the combination of high stocking densities, low quality diets (Ellis et al., 2002) and lack of management technology for native fish species could lead to disease outbreaks. Bacteria are normally present in water, ponds and fish; however, many bacteria ubiquitous in the rearing environment are opportunistic and can take advantage when an imbalance in the relationship amongst the host (fish itself), the environment and pathogen occurs (Cyrino et al., 2010). The global economic loss in aquaculture due to disease amounts to more than US$ 9 billion dollars per year (Shinn et al., 2015). Although there are no exclusively Brazilian reports, Tavares-Dias and Martins (2017) estimated, based on scientific information and official data, the loss of production in freshwater fish in Brazil reaches around 15% each year.

Diseases and subsequent economic losses caused by Gram-positive bacteria, such as bacterial kidney disease (BKD) in salmonid fish (Fryer and Sanders, 1981), streptococcosis in tilapia (*Oncorhyncus mykiss*) (Inglis et al., 1993) and clostridial zoonosis (Gauthier, 2015), are well reported in the aquaculture industry. Gram-negative bacteria are also well known agents of disease and financial loss. Such diseases include, *Edwardsiella* septicemia in warm water fish ((e.g. channel catfish (*Ictaluris punctatus*) and Japanese eel (*Anguilla japonica*) (Inglis et al., 1993)); vibriosis in salmonids (Toranzo et al., 2005) and motile aeromonad septicemia in carp (*Cyprinus carpio*) and channel catfish (*Ictaluris punctatus*) (Inglis et al., 1993). Despite this breadth of knowledge very little is currently known about bacterial etiological agents of pirarucu, *Arapaima gigas*.

Besides high mortality caused by bacteria in aquaculture, pathogenic bacterial strains can also cause internal alterations in the liver and kidney and haemorrhagic spots on the heart and brain (Silva et al., 2012). Externally, many different symptoms such as ulcerations, haemorrhages, and erosion of the fins (Dias et al., 2016) have been reported. These alterations and symptoms prevent farmers from

commercializing their fish because of degradation of the appearance of fish (i.e. loss of aesthetic qualities) and potential zoonotic risk to human health.

The species *Flavobacterium columnaris* was identified as an *A. gigas* pathogen by Ono et al. (2004), however, Dias et al. (2016) reported that pirarucu is also affected by pathogenic bacteria common in aquaculture production such as *Aeromonas hydrophila*. In the present chapter the bacterial strains were isolated from diseased pirarucu (Chapter 3a) and belong to taxa previously documented as pathogens in fish, such as *Citrobacter freundii* (Austin and Austin, 2007; Karunasagar et al., 1992) and *Pseudomonas* sp. (Attia A Galil YA, Fathi M, 2012; Colquhoun et al., 1998; Jang et al., 2014).

To date, there are no reports of these two isolated strains causing disease in pirarucu (*A. gigas*) in Brazil. The aim of this chapter was to characterise the pathogenicity of two strains *C. freundii* and *Pseudomonas* sp. as potential pathogens for the production of pirarucu.

## 3b.2 Material and methods

The bacteria used in this experiment were isolated from a disease outbreak in *Mar e Terra Ind. Com. de Pescados* and identified in Chapter 3a. The strains of *Pseudomonas* sp. (PP2) and *Citrobacter freundii* (PP3) were grown in BHI media (Oxoid, UK) for 24 h at 30 ºC. After the incubation period the concentrations were checked according standard growth curves previously established according to the methodology described by Silva et al. (2012). The strains were then centrifuged for 10 min at 4,000 g at 4 ºC and re-suspended in sterile PBS (Oxoid, UK) to achieve final concentrations of $1x10^8$, $1x10^6$ and $1x10^4$ CFU.ml$^{-1}$. Each individual fish was challenged intraperitoneally (10 µl g$^{-1}$ fish) with the bacterium at the concentrations detailed above.

**DNA extractions and PCR**

The potential pathogenic strains were grown in BHI broth for 18 h at 30˚C, centrifuged at 4,000 g for 10 min and re-suspended in TE buffer (0.5% times of the original volume). The DNA was extracted from the pellets using the CTAB method, adapted from the Joint Genomic Institute (JGI) protocol (William and Copeland 2004). For detailed information see sections 2.5 and 2.7 from Chapter 2.

**Motility**

One fresh colony, from each bacterial strain identified as potentially pathogenic, was inoculated by stabbing the middle of the semi-solid PB agar (Poor Broth medium (1% peptone, 0.5% NaCl and 0.5% agar).

**Biochemical characterization**

Biochemical profile of isolated strains was performed using API 20E V5.0 (BioMérieux, Marcy l'Etoile, France). For further information see section 2.4 from Chapter 2.

**Challenge**

A pre-infection test was performed to check the pathogenicity dose in *A. gigas* individuals. Nine fish were distributed in three tanks and infected with the *Pseudomonas* sp. or *Citrobacter freundii* at $1x10^9$ $CFU.ml^{-1}$; and a control group infected with PBS. After 24 h survival levels were checked. The group infected with *Pseudomonas* sp. had 33.3% mortality while group infected with *C. freundii* had 100% mortality and the fish infected with PBS had 0% mortality.

For the LD 50 test, a total of 112 *A. gigas* weighing an average of 15.38 ± 3.69 (g) were purchased from *Rio Doce Piscicultura* farm and transported to the facilities of AQUOS (Aquatic Organisms Health Laboratory) from the Federal University of Santa Catarina (UFSC), Brazil. The fish were randomly distributed across 14 tanks (100 l) totalling 8 fish per tank and were acclimated for 7 days. The tanks were maintained in a closed-water recirculation system with mechanical and biological filters at a constant temperature of 28°C. The fish were intraperitoneally challenged within the following concentrations, in duplicate: *Pseudomonas* sp. at $1x10^8$, $1x10^6$ and $1x10^4$ $CFU.ml^{-1}$; *Citrobacter freundii* at $1x10^8$, $1x10^6$ and $1x10^4$ $CFU.ml^{-1}$; a non-infected control was injected with PBS. Before the challenge the recirculation system was closed to avoid contamination between treatments.

After the experimental challenge, dead fish were removed every 6h, to calculate the cumulative mortality curve and to avoid cannibalism. This process was repeated until the mortalites apparently ceased. Three specimens of surviving fish from the most significant mortality group were sampled for the following analysis.

The fish were anaesthetized with 0.01% Eugenol (Vetec/Sigma-Aldrich) and blood was collected by puncture of the caudal vessel in two 3 mL syringes (21G), one containing 10% EDTA. The collected blood was used to make blood smears,

haematocrit, total blood cells counts and differential blood cells as described in chapter 2, section 2.18.

The internal organs were examined for macroscopic lesions. A sample of approximately 1g of liver was used to perform serial dilutions 1:10 in PBS following for plating out on TSA (Oxoid, UK) for total viable counts (TVC) and Cetrimid (Oxoid, UK) specific for *Pseudomonas* sp. to verify presence in the tissue. The grown colonies were re-striped on the same media and similar strains were discarded. A total of 8 strains were then subjected to DNA extractions for molecular identification.

**Histology**

Histological analysis of the posterior intestine was conducted according to the protocols described in section 2.18 from Chapter 2.

**Identification of pathogenic bacteria in tissue**

A section of 1g of the liver from the surviving fish was sampled. Total viable counts followed by DNA extraction from growth colonies were performed as explained in section 2.21 from Chapter 2.

The lethal concentration $LC_{50}$ after 72 hours of infection was calculated using Logit (P) and Probit (P) linearization of the standard curve which was calculated according Currell (2015). This method "considers the probability of individuals being in a specific state and consequential proportion of population on that state." Where *P* = proportion (or percentage, *P*%) that a drug, kills 50% of the population.

**Statistical analysis**

Data were tested for normality and the Bartlett test was used to verify homogeneity of variance, prior to Tukey analysis for difference of means. Data that did not present homogeneity of variance were submitted to non-parametric *Kruskal-Wallis test.*

## 3b.3 Results

Both strains were previously identified as described in Chapter 3a. Gram-negative rods shaped and positive motility was observed. The strain *Pseudomonas* sp. presented production of fluorescence while *C. freundii* did not present such characteristic. Biochemistry characteristics are described in (Table 3b.1).

Table 3b.1: Main characteristics of two potential pathogenic strains selected from diseased pirarucu (*A. gigas*) PP2 and PP3.

| **Characteristic** | **Strain 1 (PP2)** | **Strain 2 (PP3)** |
|---|---|---|
| Gram stain | - | - |
| Shape | Rod | Rod |
| Tissue isolated | Brain | Heart |
| Motility | + | + |
| Hemolysis | Beta | Alpha |
| Production of fluorescence | + | - |
| Colony color | Bright Glow yellow | White |
| Biochemical identification (API 20E V.50) | *Pseudomonas fluorencens* 79,8% | *Citrobacter youngae* 99,9% |
| Molecular identification | *Pseudomonas* sp. | *Citrobacter freundii* |
| GenBank number | GU113077.1 | KF245926.1 |

The mortality curve ceased after 60 h (Figure 3b.1), however, the observations continued until 72 h post challenge. The fish infected with *C. freundii* at $1x10^8$ CFU.ml$^{-1}$ had mortality of 50% while fish infected with *Pseudomonas* sp. at the same concentration displayed mortality levels of 6.25% at $1x10^8$ CFU.ml$^{-1}$ mortality. Surprisingly, control fish had mortality levels of 12.5% after PBS injection (one fish per replicate).

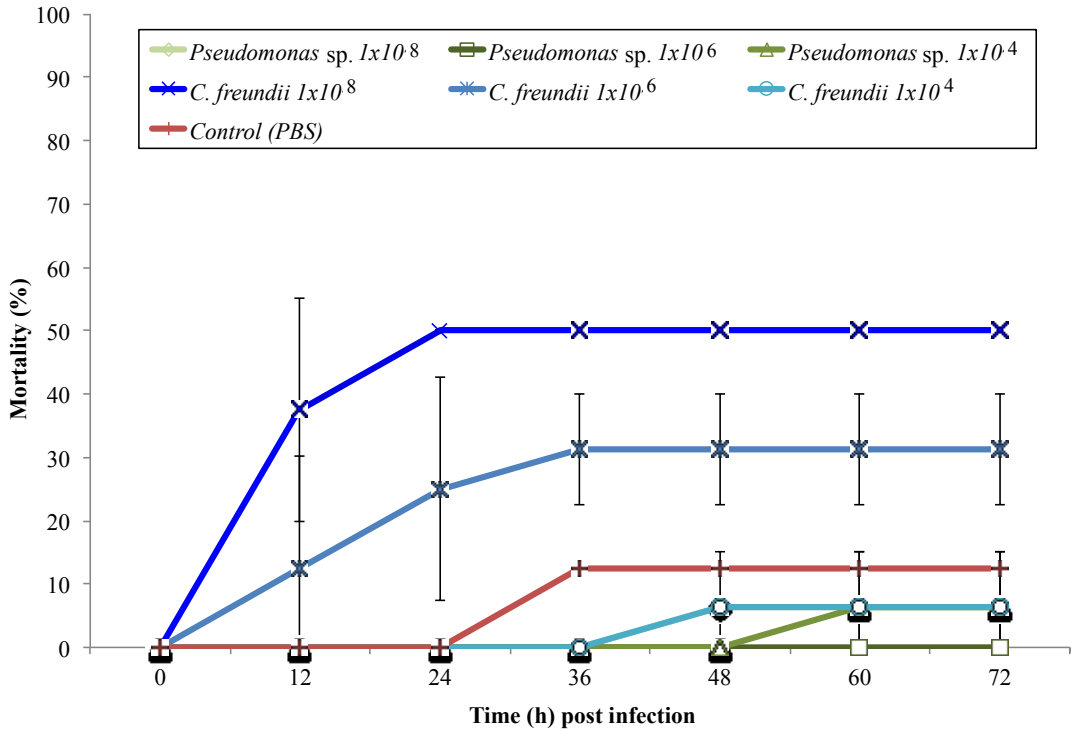



Figure 3b.9: Percentage of mortality and standard deviation of pirarucu (*A. gigas*) infected with *Pseudomonas* sp. at $1x10^8$, $1x10^6$ and $1x10^4$ CFU.ml$^{-1}$; *Citrobacter*

*freundii* at $1x10^8$, $1x10^6$ and $1x10^4$ CFU.ml$^{-1}$; and non-infected (Control) injected with PBS up to 72h post infection

Weak haemorrhagic spots were observed on the abdomen of the fish; however, severe haemorrhage was observed internally in the intraperitoneal cavity, in the brain and in the intestine. Alterations in the liver were also observed in fish infected with the strain *Citrobacter freundii* at $1x10^8$ CFU.ml$^{-1}$ after 72 hours post infection. (Figure 3b.2) The control and *Pseudomonas*-infected groups did not present any such clinical signs.

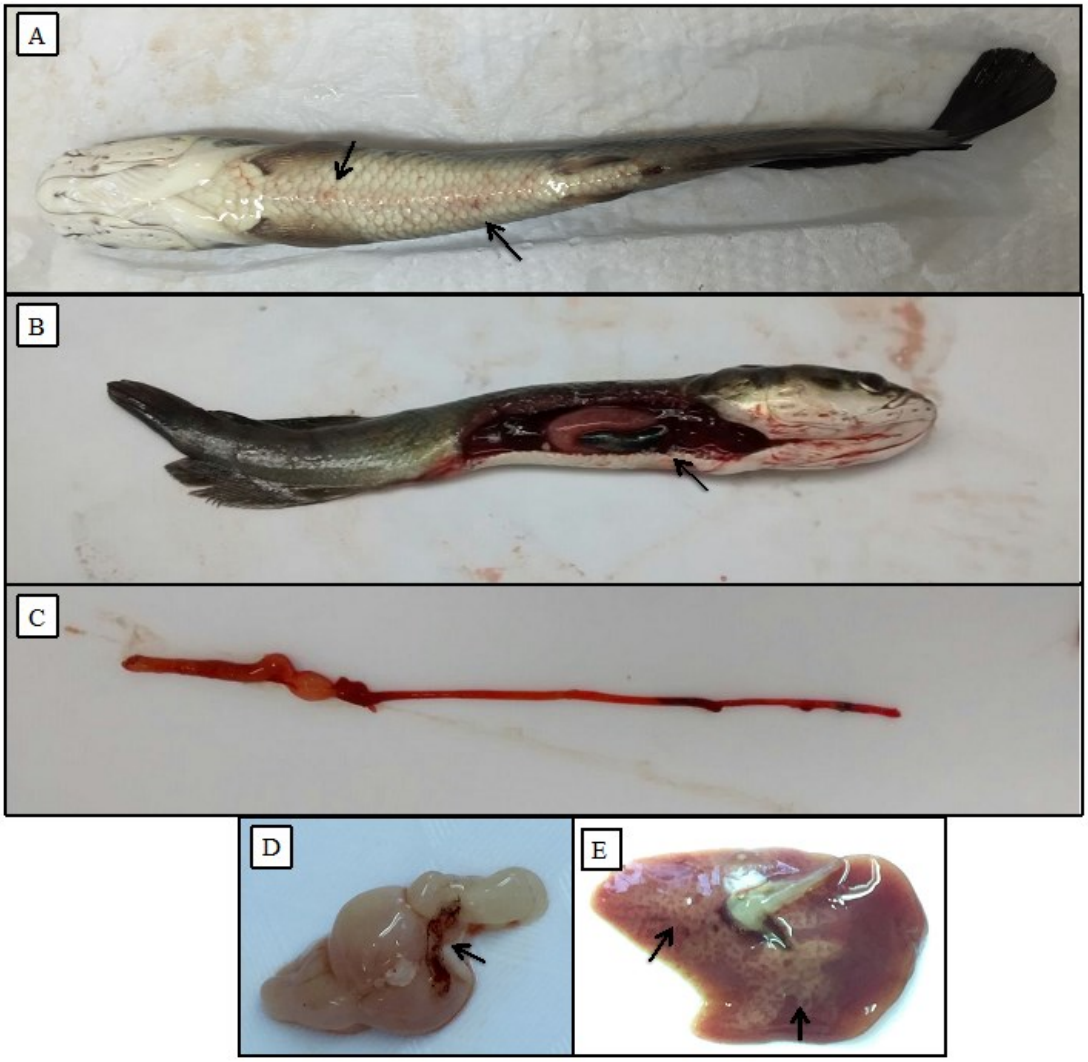



Figure 3b.10: Clinical signs of fish infected with *Citrobacter freundii* at concentration $1x10^8$ CFU.ml$^{-1}$, 72 hours after infection. Weak haemorrhagic spots on the abdomen (A), severe internal haemorrhage (B), haemorrhagic intestine (C), haemorrhagic spots in brain (D), and alterations in liver (E) are present

After 72h post infection, no difference was observed in either total viable counts (TVC) or *Pseudomonas* spp. levels between the treatments. A total of 8 different colonies were then selected for further DNA extractions and molecular

identification. Strains isolated from liver of *Citrobacter*-infected fish were identified as *Citrobacter freundii* indicating that this bacterium was able to migrate into the internal organ from the intraperitoneal cavity (Table 3b.2).

Table 3b.2: Average (± standard deviation) of total viable counts (TVC) and *Pseudomonas* sp. counts of bacteria present in liver of pirarucu (*A. gigas*) 72 h post infection with *Pseudomonas* sp. and *Citrobacter freundii*. Molecular identification was performed using16S rRNA gene and BLAST at GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

| **Strain** | **Dose (CFU.ml$^{-1}$)** | **Counts (CFU.g$^{-1}$)** | | **Molecular identification of isolates** | **GenBank Ref. Number** |
|---|---|---|---|---|---|
| | | ***Pseudomonas* sp. (1)** | **TVC (2)** | | |
| Control (PBS) | - | <1x10$^2$ * | <1x10$^2$ * | *Enterobacter* sp. | FN997633.1 |
| | | | | *Staphylococcus pasteuri* | KT728837.1 |
| *Pseudomonas* sp. | 1x10$^8$ | <1x10$^2$ * | 1.5x10$^3$ ± 0.7 | *Shewanella* sp. | KU982963.1 |
| | | | | *Chryseobacterium taeanense* | KJ794194.1 |
| *Citrobacter freundii* [1] | 1x10$^8$ | <3x10$^3$ * | 1.61x10$^6$ ± 0.3 | *Citrobacter freundii* | HQ170626.1 |
| | | | | *Citrobacter freundii* | CP016952.1 |

*Estimated from counts outside the 25-250 per plate range (Maturin and Peeler, 2001)
(1) Cetrimid medium and (2) Tryptone Soya Agar (TSA) medium

Red blood cells concentration was lower in both infected-fish groups when compared with non-infected fish (0.001) indicating anaemia in infected fish (Table 3b.3). Higher lamina propria (LP) width was observed in histological intestine samples from both infected fish (Figure 3b.3), however no statistical difference of villi length, and number of globed cells between treatments infected with the analysed strains at 1x10$^8$ CFU.ml$^{-1}$ after 72 hours post infection (Table 3b.4). The lethal concentration ($LC_{50}$) for *C. freundii* after 72 hours of infection was 2.02x10$^8$ CFU.ml$^{-1}$ and 3.88x10$^8$ for Logit (*P*) and Probit (*P*), respectively (Figures S3b.1; S3b.2 and Table S3b.2). The $LC_{50}$ for *Pseudomonas* sp. could not be calculated due to the relative uncertainties of low mortality percentage and it was considered non pathogenic for pirarucu at concentration tested.

Table 3b.3: Red blood cells (RBC), differential white blood cells (WBC) concentration of in pirarucu (*A. gigas*) 72 hours post infection with *Pseudomonas* sp., *Citrobacter freundii at* $1x10^8$ $CFU.ml^{-1}$ and control (infected with PBS)

| Treatment | RBC ($x10^6.ml^{-1}$) | Thrombocytes ($x10^5.ml^{-1}$) | Lymphocytes ($x10^5.ml^{-1}$) | Neutrophils ($x10^5.ml^{-1}$) |
|---|---|---|---|---|
| Control | 2.49±0.17[b] | 10.2±8.53 | 11.2±3.95 | 8.32±1.93 |
| *Pseudomonas* sp. | 1.69±0.08[a] | 7.13±6.01 | 4.35±1.64 | 9.82±3.96 |
| *Citrobacter freundii* | 1.88±0.14[a] | 2.09±1.49 | 5.60±3.47 | 8.74±5.21 |

| Treatment | Monocytes ($x10^5.ml^{-1}$) | Young cells ($x10^4.ml^{-1}$) | Haematocrit (%) | Eosinophils ($x10^5.ml^{-1}$) |
|---|---|---|---|---|
| Control | 5.16±2.46 | 2.58±2.58 | 34.16±2.92 | 0.00±0.00 |
| *Pseudomonas* sp. | 2.15±1.97 | 0.00±0.00 | 34.00±8.04 | 0.00±0.00 |
| *Citrobacter freundii* | 4.87±3.80 | 0.00±0.00 | 38.33±0.76 | 0.00±0.00 |

Table 3b.4: Histological parameters of posterior intestine of in pirarucu (*A. gigas*) 72 hours post infection with *Pseudomonas* sp., *Citrobacter freundii at* $1x10^8$ $CFU.ml^{-1}$ and control (infected with PBS)

| Treatment | Villi Length | LP width | Goblet cells |
|---|---|---|---|
| Control | 236.77±99.74 | 26.89±4.96[a] | 13.46±3.32 |
| *Pseudomonas* sp. | 247.63±89.85 | 44.41±11.41[b] | 14.18±7.94 |
| *Citrobacter freundii* | 296.08±61.85 | 43.33±18.28[b] | 23.29±6.01 |

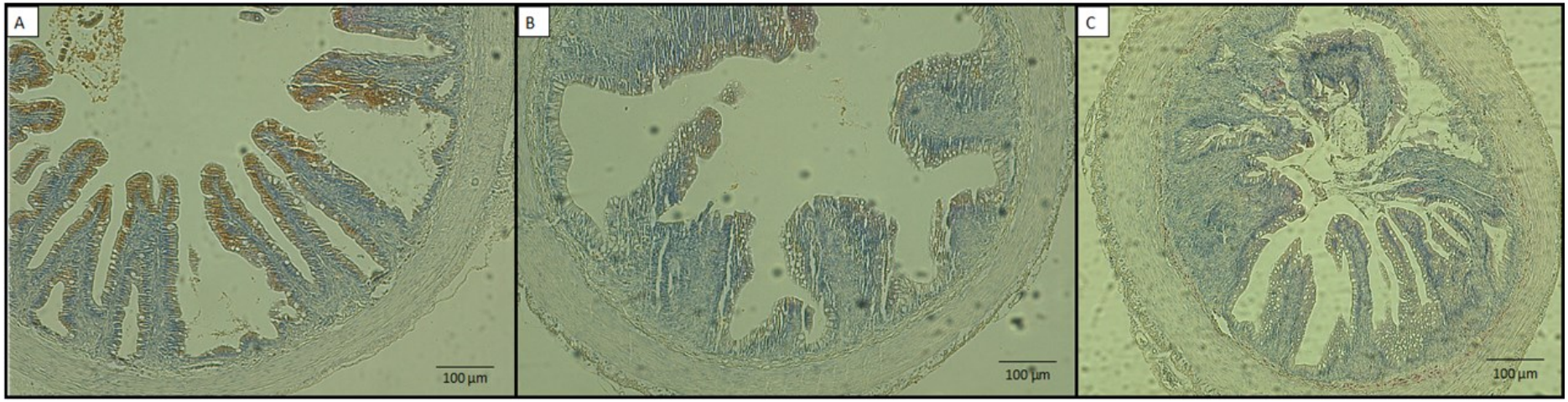


Figure 3b.3: Light microscopy of posterior intestinal morphology of in pirarucu (*A. gigas*) 72 hours post infection with *Pseudomonas* sp. (b), *Citrobacter freundii* (c) *at* $1x10^8$ $CFU.ml^{-1}$ and control (a) (infected with PBS). All pictures were taken on 10X magnification

## 3b.4 Discussion

In recent years, pirarucu (*Arapaima gigas*) production has increased substantially compared to other native fish species in Brazil. Due to this growth there is a concern about possible pathogenic bacteria that could affect production causing production loss and possible zoonosis. The present study has characterized two bacterial stains previously isolated from outbreaks in pirarucu production farms in Rondônia state in Brazil.

*Citrobacter freundii* was previously reported as being pathogenic for different fish species (Gallani et al., 2016; Jeremić et al., 2003; Lü et al., 2011; Thanigaivel et al., 2015). In addition, bacteria from Pseudomonadaceae family such as *Pseudomonas fluorescens* is able to cause of septicaemia in fish (Gauthier, 2015; Inglis et al., 1993). In the present study, the *C. freundii* strain presented signs of pathogenicity, however, the tested concentrations for *Pseudomonas* sp. did not have a pathogenic effect.

Although the strain PP2 isolated in the present study presented some pathogenic characteristic such as positive haemolysis and phenotypical sugar fermentation similar to *Pseudomonas fluorescens* (79,8%), its virulence seems not to affect *A. gigas* at the doses tested. *Pseudomonas* spp. are normally present in soil and water (Gauthier, 2015), and are easily isolated because they grow aerobically in non-selective media such as tryptone soya agar (TSA), brain heart infusion (BHI), and nutrient agar (NA) (Inglis et al., 1993). Thus, the fact they were isolated from diseased fish doesn't attest its pathogenicity. For instance, according Koch's postulates, the microorganism should be re-isolated from diseased fish and further identified to be confirmed as pathogenic (Evans, 1976).

Regardless extreme care was taken during the infection management; the mortality in control group could be explained by handling stress. The two dead fish, in this case, was computed under control group mortalities and properly added on the $LD_{50}$ calculations as casual death (see Table S3b.2).

The $LD_{50}$ represents the concentration of bacteria causes 50% mortality within a population. In the present study, *A. gigas* from the group infected with $1x10^8$ $CFU.ml^{-1}$ displayed 50% mortality. This concentration was confirmed by probability linear model Logit (*P*) and Probit (*P*). Pathogenicity tests performed by Thanigaivel et al. (2015) using strains of *C. freundii* in tilapia (*Oreochromis mossambicus*) showed

that $LD_{50}$ of this bacteria after 72 hours of intramuscular injection was $2.35x10^5$ $CFU.ml^{-1}$ and $3.4x10^6$ $CFU.ml^{-1}$ for immersion infection. In contrast, zebrafish (*Danio rerio*) infected by immersion at approximately $10^8$ $CFU.ml^{-1}$, had 66.7% of mortality up to 7 days of infection (Lü et al., 2011). The $LD_{50}$ presented by Thanigaivel et al. (2015) are lower than the dose of the present study, however, the exposure challenge methods used (immersion and intramuscular injection) were different from the ones used at the present study (IP injection). Indeed, pathogens find different routes to infect the host with the main portals of entry being the skin, gills, and gastrointestinal tract (Merrifield et al., 2010; Ringø et al., 2010). Thus, these different routes influence the mortality data as well as clinical sings in experimental infections for pathogen characterization. Bacteriosis could also depend on factors such as virulence of the bacteria and inoculation temperature (Dias et al., 2016). In addition, different fish species may have different response of immunity to pathogens and the $LD_{50}$ could vary among them. Further studies should be performed using specific host pathogens in Brazilian native fish to permit the development of diagnosis and possible treatments for bacterial diseases.

Although fish liver should be normally be devoid of live bacteria, isolates were obtained from the liver of fish that were not infected (control). These were identified as *Staphylococcus pasteuri* and *Enterobacter* sp. Strains of *Enterobacter* sp. are normaly found in fish pond water (Miruka et al., 2013) and strains of *S. pasteuri* were described to be indigenous from posterior gastrointestinal tract of Atlantic salmon (*Salmo salar*) (Askarian et al., 2012). Thus, the fish mortalities in the group infected with PBS should not be related with the presence of these two strains in their liver. On the other hand, two different strains *Shewanella* sp. and *Chryseobacterium taeanense* were identified in fish infected with *Pseudmonas* sp.. Strains of *Shewanella putrefaciens* and *Shewanella baltica* were previously used as probiotics in *Senegalese sole* (*Solea senegalensis*) (García de La Banda et al., 2010; Sáenz de Rodrigáñez et al., 2009), thus they were not likely to be primary pathogens for the fish. Likewise, to the author's knowledge, there is no report of *C. taeanense* infections in fish, thus this strain may have been isolated due to contamination during the isolation procedure.

In the present study, strains of *C. freundii* isolated from the liver of infected fish challenged with this same species revealed that this strain was able to migrate from intraperitoneal cavity and infect the liver causing clinical alterations. Thus, *Citrobacter freundii* clinical signs observed corroborate the signs described in other

fish species infected with this same bacterium. Haemorrhages on the skin and fins was observed in cyprinids (Jeremić et al., 2003), and grass carp (Lü et al., 2011) infected with *C. freundii*. Brain haemorrhages and intestinal oedema were also observed in *C. freundii* infected South American catfish (*Pseudoplatystoma* sp.) (Pádua et al., 2014). Severe gill damage observed previously in tilapia and other cyprinids (Jeremić et al., 2003; Thanigaivel et al., 2015) was not observed in pirarucu infected with *C. freundii* In the present study.

The significant decrease of peripheral red blood cells levels in pirarucu infected with both bacteria *C. freundii* and *Pseudomonas* sp. when compared with the control group suggested anaemia in infected fish. Indeed erythrocytes number observations at the present study was also lower in when compared with pirarucu under "normal" condition (Tavares-Dias et al., 2007). Anaemia was also related with decrease of erythrocytes in South American catfish (*Pseudoplatystoma* sp.) infected with *Aeromonas hydrophila* at different concentrations when compared with non-infected fish (Silva et al., 2012). The decrease in the number of red blood cells in fish can be related to a decrease in oxygen carried by haemoglobin, affecting fish physiology and causing stress (Sloman et al., 2000).

## 3b.5 Conclusion

This is the first study that has assessed *C. freundii* and *Pseudomonas* sp. pathogenicity in *A. gigas*. The $LD_{50}$ dose for *C. freundii* infections was calculated. This is useful information for Brazilian pirarucu farmers and fish veterinarians, and the clinical signs caused by *C. freundii* described in this chapter may help to support improved diagnosis during possible mortality outbreaks in pirarucu farms in Brazil.

# Chapter 4:
# Ability of autochthonous probiotics to modulate intestinal microbiota of pirarucu, *Arapaima gigas*

# CHAPTER 4: Ability of autochthonous probiotics to modulate intestinal microbiota of pirarucu, *Arapaima gigas*

## 4.1 Introduction

The environment and host characteristics have been reported as important factors having a key role in impacting the abundance and activity of fish gut microbiota, however, the diet is generally considered to be the major driving factor (Ringø et al., 2016). The capacity to adhere to the intestinal tract and be "viable" within the intestinal mucus is an important characteristic of bacterial probiotic candidates (Merrifield et al. 2010). For this reason, the *in vivo* study of mucosal colonisation and general gut microbiota modulation driven by probiotic candidates is essential to affirm probiotic characteristics of a bacterial strain.

Probiotics are linked to a modulation of gut microbiota in many fish species. The impact of the probiotic on the gut microbiota can lead to many of different outcomes, such as increase of LAB levels (Ferguson et al., 2010; Jatoba et al., 2011; Standen et al., 2015), increase of total viable counts (Ridha and Azad, 2012), decrease of total viable counts (Jatoba et al., 2011), decreased of pathogens levels (Del'Duca et al., 2013) and alteration of microbial diversity (Ramos et al., 2013). These diverse, and often inconsistent or conflicting, effects are a result of a complex interaction between different resident microbiota present in different fish species, different probiotic feeding regimes and different fish rearing conditions. In fact, a continued probiotic feeding for long periods is required to maintain and possibly implant a probiotic population in fish gut; increasing this way a higher probability viability up to 2-3 weeks after cessation of feeding (Merrifield et al., 2014).

However, many studies that have assessed fish gut microbiota after being fed with probiotics have used culture-based tools. Molecular culture-independent technologies such as the HTS have being currently used to assess fish gut microbiota. The use of HTS analysis is a novel alternative that offers a high resolution approach to analyze the microbial diversity and interactions that can be used to understand intestinal bacterial communities in fish (Ju and Zhang, 2015). After understanding the host intestinal communities, specific studies could be applied to increase fish health and nutrition requirements.

The aim of the present chapter was to analyse the potential of two autochthonous bacteria (*L. lactis* subps. *lactis* and *E. faecium*) to colonise and modulate the pirarucu (*Arapaima gigas*) gut microbiome. Subsequently, the impact of potential modulation on gut morphology and benefits on immunological and haematological parameters were assessed.

## 4.2 Material and Methods

### 4.2.1 Biological Material

The strains *Lactococcus lactis* subsp. *lactis* and *Enterococcus faecium* were isolated and identified in chapter 3. The strains were activated by inoculating 1 colony in 9 ml MRS (Oxoid, UK), and incubating at 35 ˚C for 24-48 h.

One hundred and thirty five *A. gigas* weighing an average of 58.86 ± 10.25 g removed from an excavated pond of *Mar e Terra Ind. Com. de Pescados* fish farming (Rondonia state, Brazil) and randomly placed into three tanks (1000 l) totalling 45 fish per tank. The tanks were placed in a flow-through system fed by a main weir. The water quality parameters were measured every two days to assure water quality. Dissolved oxygen (5.27 ± 0.35 $mg.l^{-1}$) and water temperature (27.4 ± 1.53 °C) were measured using optical dissolved oxygen instrument (YSI, USA). The water pH (6.56 ± 0.32) and concentration of ammonia (0.91 ± 0.27 $mg.ml^{-1}$), and nitrite (0.02 ± 0.01 $mg.ml^{-1}$) were measured using a kit for farmers to fresh water (Alfakit, SP, Brazil).

### 4.2.2 Experimental design

Fish were fed at 6% of biomass over four feeds per day (following the management protocols of the farm). The experiment lasted 21 days and the treatments were as follows: (1) fish fed commercial diet supplemented with *L. lactis* subps. *lactis*, (2) fish fed commercial diet supplemented with *E. faecium*, and (3) fish fed commercial diet without supplementation. An extruded commercial diet *Do Peixe Revolution Alevino 2 to 3 mm* (Douramix, Brazil) which contained 40% crude protein and 11% crude lipid was used during the experiment.

### 4.2.3 Inoculation of probiotic bacteria

Both probiotic bacteria *L. lactis* subsp. *lactis* and *E. faecium* were grown in MRS broth (Oxoid, UK), and incubated at 35°C for 48h. The suspension was centrifuged at 4,000 g for 15 min and re-suspended in PBS (tablets Oxoid, UK). After

that, 100 mL of the probiotic at concentration of $1x10^9$ CFU $mL^{-1}$ was sprayed (using a sterile plastic spray bottle) on each kilogram of the commercial diet. The diet was mixed with the probiotic by shaking the bag by hand for 5 min to ensure even coverage and then dried at 30°C for 12h. This process was repeated every week to achieve the same probiotic concentration in the diet.

To establish the probiotic concentration in the diet, 1g of diet was homogensed in a sterile mortar with 1 ml of PBS and then serially diluted in vials at 1:10 factor. The dilutions $10^{-5}$ to $10^{-9}$ were plated in petri dishes containing MRS agar with 1% of aniline blue 1% (v/v). The plates were incubated at 35°C for 48h. This process was repeated every time the diet with probiotic was prepared. The probiotic concentration in the diets were 3.00 ± 2.33 $x10^8$ CFU.$ml^{-1}$ and 2.92 ± 3.14 $x10^8$ CFU.$ml^{-1}$ for *L. lactis* subps. *lactis* and *E. faecium,* respectively.

### 4.2.4 Sampling

After the experimental period, 12 fish from each tank were sampled as biological replicates, anesthetized with 0.01% Eugenol (Vetec/Sigma-Aldrich), and terminated by destruction of the brain. The fish were externally washed with ethanol 70%, then, a cut was made on the abdomen using a sterile scalpel blade. The posterior intestines were aseptically excised using sterile forceps, and the digesta (intestinal content) and mucosa (intestinal tissue) were sampled. Mucosa samples were washed with sterile phosphate-buffered saline (PBS Oxoid, UK).

Both mucosa and digesta samples were stored in DNA free vials 98% molecular grade ethanol (Sigma-Aldrich). Prior to the DNA extractions, all samples were centrifuged (17.000 g for 5min) to remove the ethanol and ~100 mg samples were used to extract the DNA as described elsewhere (Falcinelli et al., 2015).

To perform HTS analysis the extracted DNA from all samples were used as template for PCRs using primers 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 338R-I (5' GCW GCC TCC CGT AGG AGT 3'), 338R-II (5' GCW GCC ACC CGT AGG TGT 3'). The PCRs were performed according to Gajardo et al. (2016). PCR products were purified (QIAquick PCR Purification Kit; Qiagen) and quantified using a Qubit® 2.0 Fluorometer (Invitrogen). Before sequencing the amplicons were assessed for fragment concentration as described by Falcinelli et al. (2015). All taxonomic analyses were performed after the removal of low quality scores (Q < 20)

with FASTX-Toolkit (Hannon Laboratory, USA). Sequences were concatenated and sorted by sequence similarity into a single fasta file, denoised and analyzed using QIIME 1.8.0 (Caporaso et al., 2010b). The USEARCH quality filter pipeline (Edgar, 2010) was used to filter out putative chimeras and noisy sequences and carry out operational taxonomic unit (OTU) picking on the remaining sequences. The taxonomic affiliation of each OTU was determined based on the Greengenes database (DeSantis et al., 2006) using the RDP classifier (Wang et al., 2007) clustering the sequences at 97% similarity with a 0.80 confidence threshold and a minimum sequence length of 300 base pairs. Non-chimeric OTUs were identified with a minimum pairwise identity of 97%, and representative sequences from the OTUs were aligned using PyNAST (Caporaso et al., 2010a). To estimate bacterial diversity, the number of OTUs present in the samples was determined and a rarefaction analysis was performed by plotting the number of observed OTUs against the number of sequences. Additionally, Good's coverage, Shannon-Wiener (diversity) and Chao1 (richness) indices were calculated.

The blood was collected by puncture of the caudal vessel in two 3 mL syringes (21G), one containing 10% EDTA was used to make blood smears which were subsequently stained with Giemsa/ May Grunwald staining (Rosenfeld, 1947) for differential leukocyte counts, as well as to obtain the total number of leukocytes and thrombocytes. An aliquot of 20µl was diluted in 980µl Dacies solution to quantify the total number of erythrocytes (RBC) in haemocytometer.

The second vial of blood was stored overnight at 4 °C to allow for coagulation, followed by and centrifugation at 1400 g for 10 min to obtain the serum witch was stored at -20 °C. The lysozyme activity of the serum was determined using the methodology adapted by Ellis (1990). A suspension of 0.02% (w/v) of *Micrococcus lysodeikticus* (Sigma-Aldrich, USA) in PBS was prepared immediately before it was used. A volume of 20 µl of serum, in five replicates, was inoculated into flat bottom microplate, and 200 µl of the suspensions of *M. lysodeikticus* cells was added to each well. The initial and final absorbance (after 10 min at 35 °C) of the samples were measured in a microplate reader (Expert Plus Asys®) at 492nm and the rate of reduction in absorbance of the samples was converted to lysozyme concentration (µg $mL^{-1}$) determined by the standard curve previously made with lysozyme from chicken egg whites (HEWL; Sigma-Aldrich).

Serum antimicrobial activity was tested against: *Pseudomonas* sp. and

*Citrobacter freundii*. For detailed methodology please see chapter 2 section 2.19.

A portion (ca. 1 cm in length) from the posterior intestines of four fish per treatment was sampled and fixed in 10% formalin for 76 h and then placed in 70% ethanol for storage. Samples were then treated with 10% formalin for 5 min; dehydrated in a graded ethanol series (50% IMS for 2.5 h, 70% IMS for 2.5 h, 90% IMS for 2 h, 100% IMS for 2h, 100% IMS for 1,5 h, 100% IMS for 1,5h); filled with wax (2x wax for 2h) and embedded in paraffin wax using a Leica EG1150H.

Three to four sections of 6 μm thick from each sample (wax block) were cut using a microtome (Leica), placed onto glass slides and left to dry overnight. The slides were then staining using an Alcian Blue-PAS (AB-PAS) protocol. Briefly, the slides were hydrated in histolene for 5 min and in 100% IMS for 5 min, placed in 1% Alcian Blue for 20 min, oxidized with 1% Periodic Acid for 10 min, placed in Schiff reagent for 3 min, stained lightly with haematoxylin for 90 sec and blue in Lithium Carbonate <10 seconds. Between each change of reagent the slides were washed with running tap water. After that, the slides were placed in 100% IMS and histolene for 1 min in each one, coversliped using DPX and left to dry overnight.

Photographs were taken using Leica digital microimaging device (DMD108, Leica Microsystems) and analyzed using imageJ v1.4r. Three different globlet cells: acidic mucins (bright blue), neutral mucins (magenta), and both acidic and neutral mucins (blue-purple or purple) were observed and separately quantified within a distance of 100 um from the top of each fold. A number of 6 to 8 folds was analyzed for each sample and also measured the LP width and length. The average of each sample was annotated for further statistical analysis.

### 4.2.5 Statistical analyses

Data were tested for normality and the Bartlett test was used to verify homogeneity of variance, prior to Tukey analysis for difference of means. Data that did not present homogeneity of variance were submitted to non-parametric *Kruskal-Wallis* test. High-throughput sequencing data was analyzed using Primer V6.0 software (PRIMER-E Ltd., Ivybridge, UK) with PERMANOVA + (Segata et al., 2011). The permutation analysis were performed with 999 permutations to the weighted and unweighted UniFrac distance matrix from beta diversity analysis in QIIME within significance at $p<0.05$. The similarities between the microbiota compositions of the intestinal samples from the two fish sizes investigated were

compared using weighted principal coordinate analysis (PCoA) and unweighted pair group method with arithmetic (UPGMA). Liner discriminant analysis (LDA) effect size (LEfSe) was used to verify significant differences in OTUs among treatments using the online interface available at http://huttenhower.sph.harvard.edu/galaxy. LEfSe analysis was determined using Alpha value of 0.01 for both Kruskal-Wallis test and parwise Wilcoxon test; and threshold on the logarithmic LDA score was kept as default (2.0) as well as the strategy for multi-class analysis (all-against-all).

## 4.4 Results

High throughout sequencing (HTS) of bacterial 16S rRNA V1-V2 regions from the Ion Torrent® PGM resulted in 1,572,641 reads after trimming and removal of low quality reads. *Streptophyta* reads were considered a contaminant from chloroplasts in feed materials and were then removed and filtered (at 0.005%) (Gajardo et al. 2016) resulting in 1,563,021 reads, of which 236,997 and 277,060 was assigned for the groups control digesta (ConD) and control mucosa (ConM); 229,481 and 278,042 for *Lactococcus*-fed fish digesta (LacD) and mucosa (LacM); 255,883 and 285,558 for *Enterococcus*-fed fish digesta (EntD) and mucosa (EntM). The total number of operational taxonomic units (OTUs) assigned from all groups was 271.

Alpha diversity parameters results reveal statistical differences (p=0.05) between some groups (Table 4.1). Chao1 and number of observed species (Figure 4.1) were lower in mucosa from *Entereococcus*-fed fish than in mucosa from control fish. However, control fish mucosa was more phylogenetic diverse (PD) than *Lactococcus*-fed fish and *Enterococcus*-fed fish mucosa.

Table 4.1: Alpha parameters results (Chao1 index, Observed Species, and PD Whole Tree) of intestinal microbiota composition of pirarucu (*A. gigas*) fed with probiotic *L. lactis* subsp. *lactis* mucosa (LacM) and digesta (LacD); fed with probiotic *E. faecium* (EntM) and digesta (EntD), and pirarucu without probiotic feeding mucosa (ConM) and digesta (ConD)

| Treatment | Chao1 | Observed species | Phylogenetic diversity (PD) |
|---|---|---|---|
| ConD | 192.99±23.04$^{ab}$ | 165.68±10.82$^{ab}$ | 4.25±0.39$^{ab}$ |
| LacD | 214.36±20.67$^{ab}$ | 193.17±31.22$^{ab}$ | 4.96±0.93$^{ab}$ |
| EntD | 206.09±21.93$^{ab}$ | 177.55±30.94$^{ab}$ | 4.68±0.63$^{ab}$ |
| ConM | 203.64±14.82$^{b}$ | 181.8±15.25$^{b}$ | 4.72±0.35$^{b}$ |
| LacM | 175.36±9.89$^{ab}$ | 158.38±8.71$^{ab}$ | 3.94±0.27$^{a}$ |
| EntM | 174.61±6.82$^{a}$ | 151.35±5.65$^{a}$ | 3.68±0.19$^{a}$ |

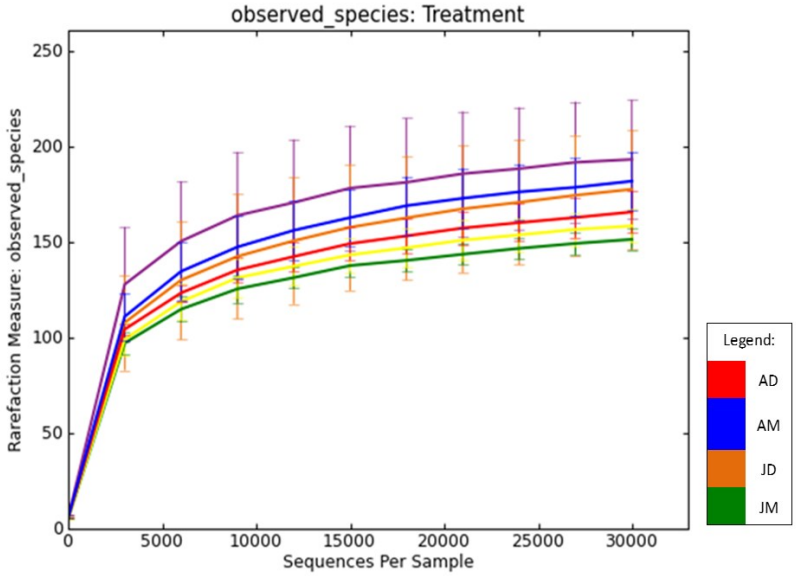



Figure 4.1: Rarefaction curve for observed species (OTUs) for pirarucu (*A. gigas*) fed with probiotic *L. lactis* subsp. *lactis* mucosa (LacM) and digesta (LacD); fed with probiotic *E. faecium* mucosa (EntM) and digesta (EntD); and pirarucu without probiotic feeding mucosa (ConM) and digesta (ConD)

Fusobacteria and Firmicutes phyla together comprised more than 90% of the total bacterial proportion in all treatments (Table 4.2). Although no significant difference was observed in their abundance between treatments groups. Firmicutes percentage in *Lactococcus*-fed fish mucosa (LacM) and *Enterococcus*-fed fish mucosa (EntM) covered 10.42% ± 8.08 and 9.67% ± 3.02 being less representative than in other treatments.

Table 4.2: Percentage of abundance (± standard deviation) at phylum level of intestinal microbiota of pirarucu (*A. gigas*) fed with probiotic *L. lactis* subsp. *lactis* mucosa (LacM) and digesta (LacD); fed with probiotic *E. faecium* (EntM) and digesta (EntD), and pirarucu without probiotic feeding mucosa (ConM), and digesta (ConD)

| | **Other*** | **Bacteroidetes** | **Firmicutes** | **Fusobacteria** | **Proteobacteria** |
|---|---|---|---|---|---|
| ConD | 0.01±0.02 | 0.09±0.07 | 36.24±20.82 | 63.65±20.83 | 0.02±0.01 |
| LacD | 0.25±0.31 | 0.08±0.06 | 24.99±19.37 | 74.6±19.7 | 0.08±0.06 |
| EntD | 0.05±0.08 | 0.06±0.06 | 15.69±10.86 | 84.13±11.03 | 0.07±0.13 |
| ConM | 0.38±0.7 | 5.19±7.34 | 26.52±21.46 | 67.57±24.65 | 0.34±0.68 |
| LacM | 0.01±0.01 | 0.75±0.67 | 10.42±8.08 | 88.78±7.76 | 0.04±0.04 |
| EntM | 00.0±00.0 | 1.71±3.61 | 9.67±3.02 | 88.58±3.55 | 0.04±0.04 |

*Other: assigns the reads lower than 0.01 (Actinobacteria, OD1 and Cyanobacteria) and non-identified phylum (Other).

The presence of the OTUs assigned as probiotic bacteria *Enterococcus* spp. and *Lactococus* spp. was confirmed after HTS analyses of fish intestine from their respective treatments, while in control groups both strains were not established (Figure 4.2a). The abundance of *Enterococcus* spp. in pirarucu intestine of groups fed with *Enterococcus faecium* was significant higher (2.47% and 1.76% for digesta and mucosa respectively), when compared with its abundance in fish intestine from other treatments. Indeed, it was the fourth most abundant genus for both *E. faecium*-fed fish treatments. On the other hand, *Lactococus* sp. abundance was not different among the groups.

At the genus level, a high abundance of *Cetobacterium* was observed in all treatments ranging from 62% to 88% of total reads (Figure 4.2b). In control fish, the second and third most abundant genus was *SMB53* (19.95% and 12.42%) and Clostridiales (order) (13.40% and 9.40%) for digesta and mucosa respectively. On the other hand, Enterococcaceae (family) (4.83% and 3.35%) and *SMB53* (4.83% and 2.50%) appeared as second and third most abundant genus in digesta (EntD) and mucosa (EntM) respectively in *Enterococcus*-fed fish; while in *Lactococcus*-fed fish the second and the third most abundant bacteria was Clostridiales (order) (7.55% and 5.01%) and SMB53 (6.21% and 3.62%) for digesta and mucosa respectively.

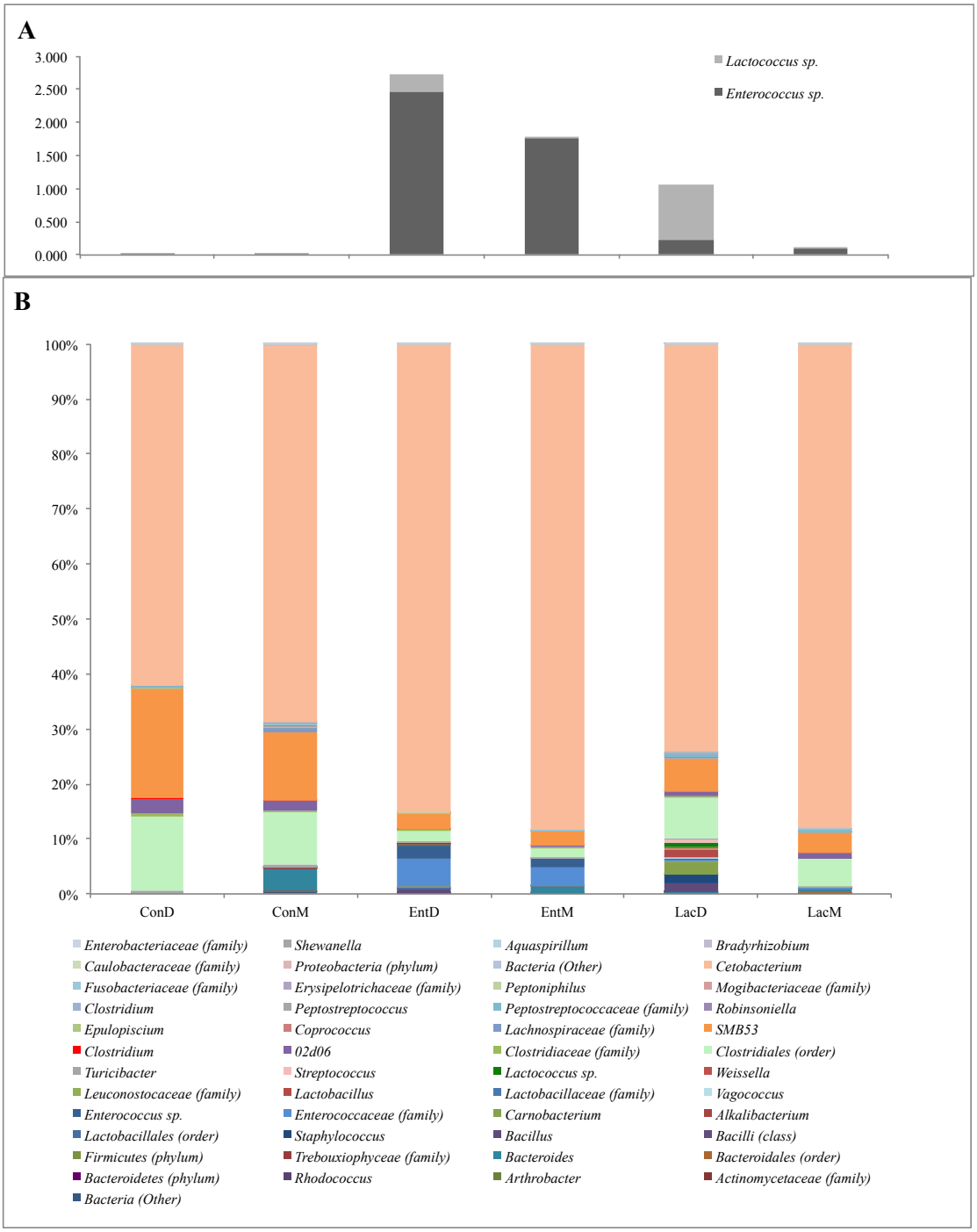



Figure 4.2: Mean *Enterococcus* and *Lactococcus* relative abundance (%) (A), and total taxonomic composition average (%) of the at genus level (B). The plots represent the abundance in each category of pirarucu (*A. gigas*) fed with probiotic *L. lactis* subsp. *lactis* mucosa (LacM) and digesta (LacD); fed with probiotic *E. faecium* mucosa (EntM) and digesta (EntD); and pirarucu without probiotic feeding mucosa (ConM) and digesta (ConD)

Linear discriminant analysis (LDA) effect size (LEfSe) results (Figure 4.3 a and b) illustrate that the class Clostridia was significant different in control fish digesta, while in control fish mucosa the difference was led by class Bacteroidia and the order Turicibactelares. The OTUs that represent the *Lactococcus* and *Enterococcus* genera were significant different in digesta in their respective treatments, however, no difference of this genus was detected in mucosa samples of this two treatments. Differential features plots (Figure 4.4) illustrate the main differences found between treatments.

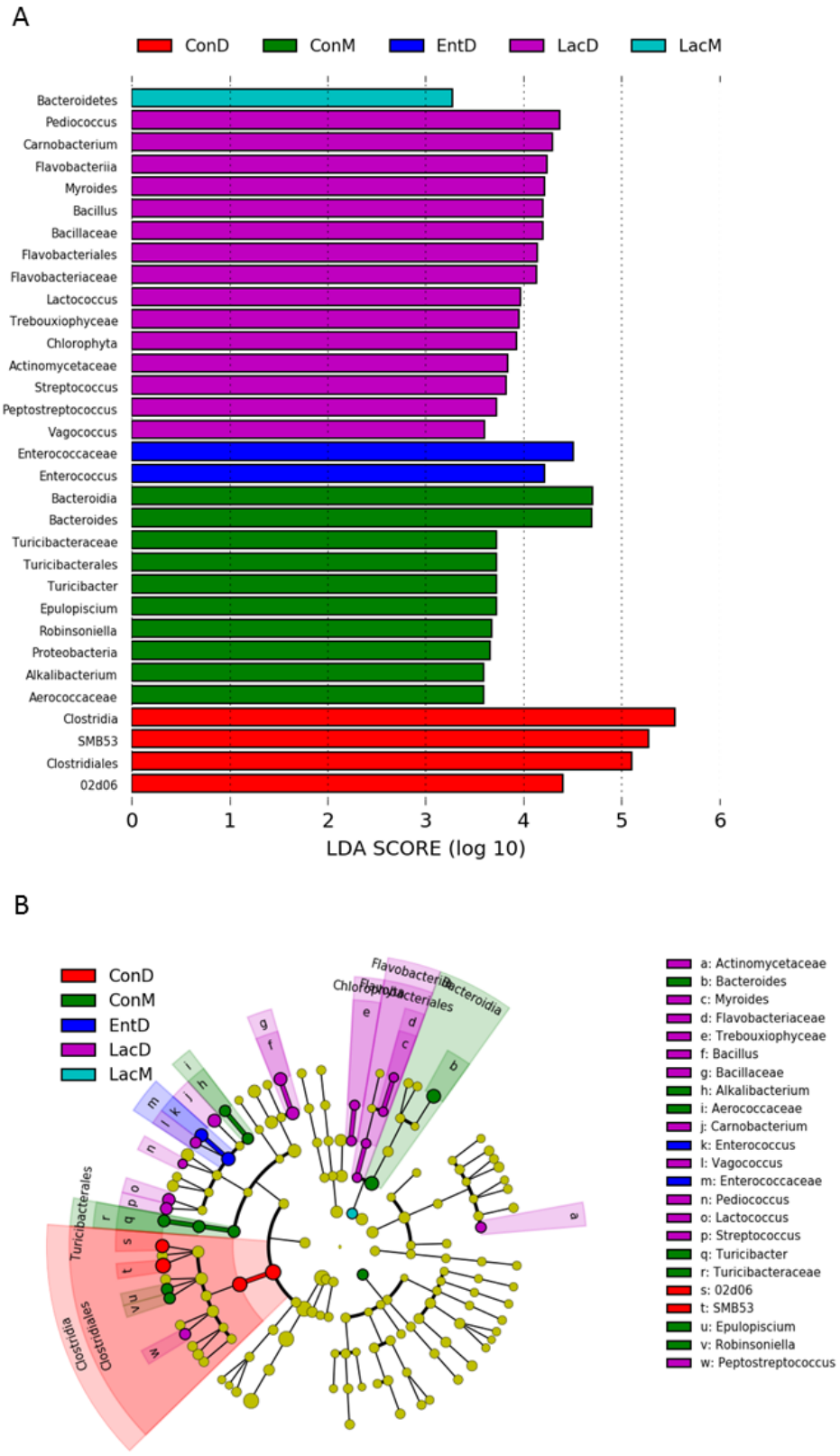



Figure 4.3: LDA (A) and cladogram plot LEfSe (B) showing the OTUs distribution around the circle for the treatments: Control digesta (ConD) and mucosa (ConM); *L. lactis* subsp. *lactis* digesta (LacD) and mucosa (LacM); and *E. faecium* digesta (EntD). Colours represent the most abundant bacteria in each category (yellow = no difference detected). *No difference was detected in *E. faecium* mucosa

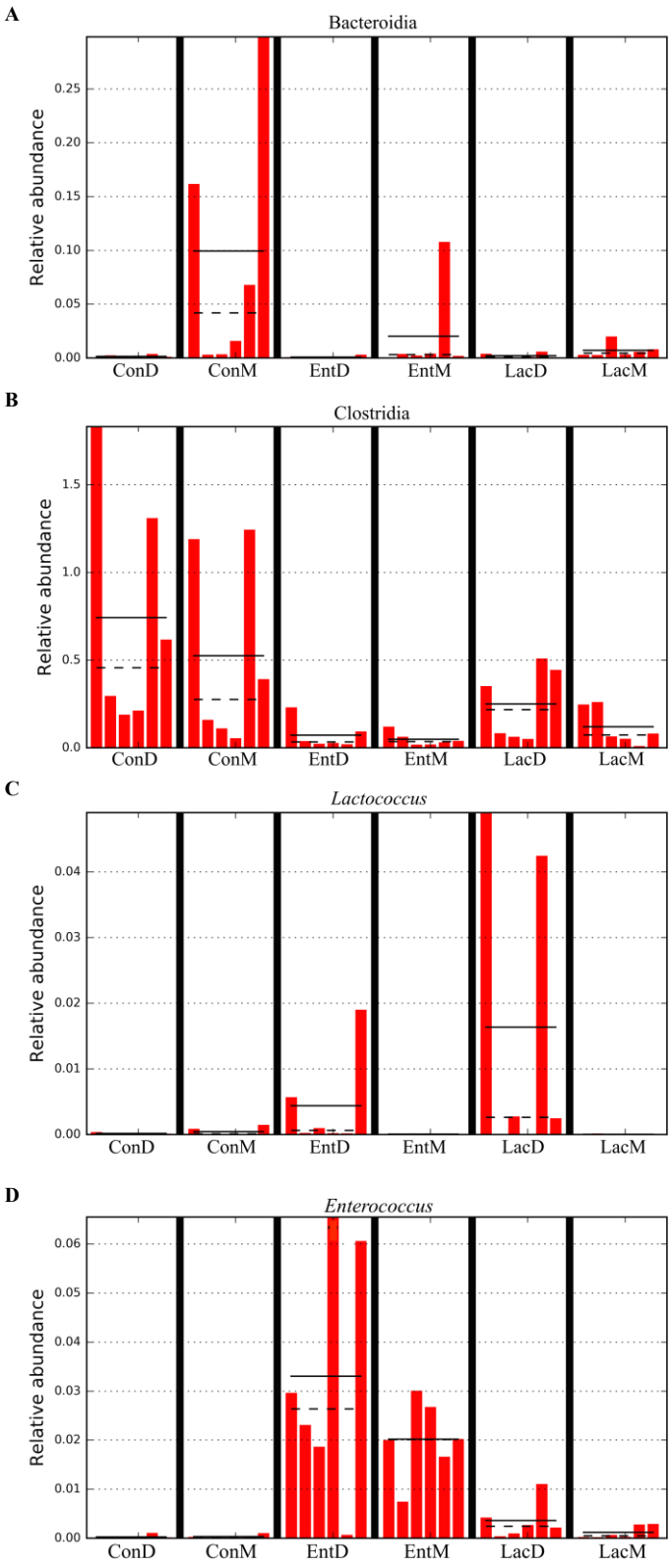
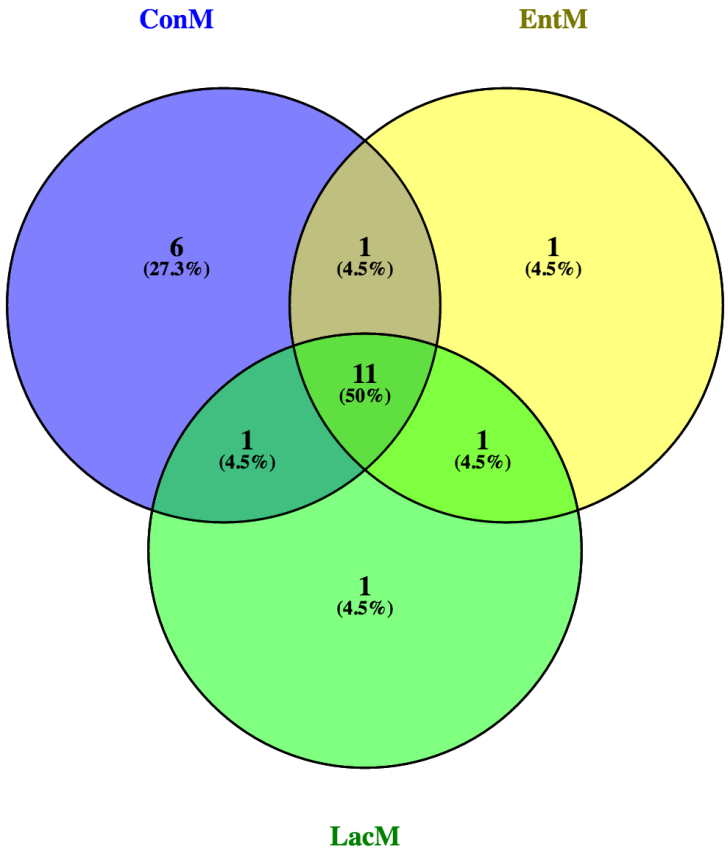


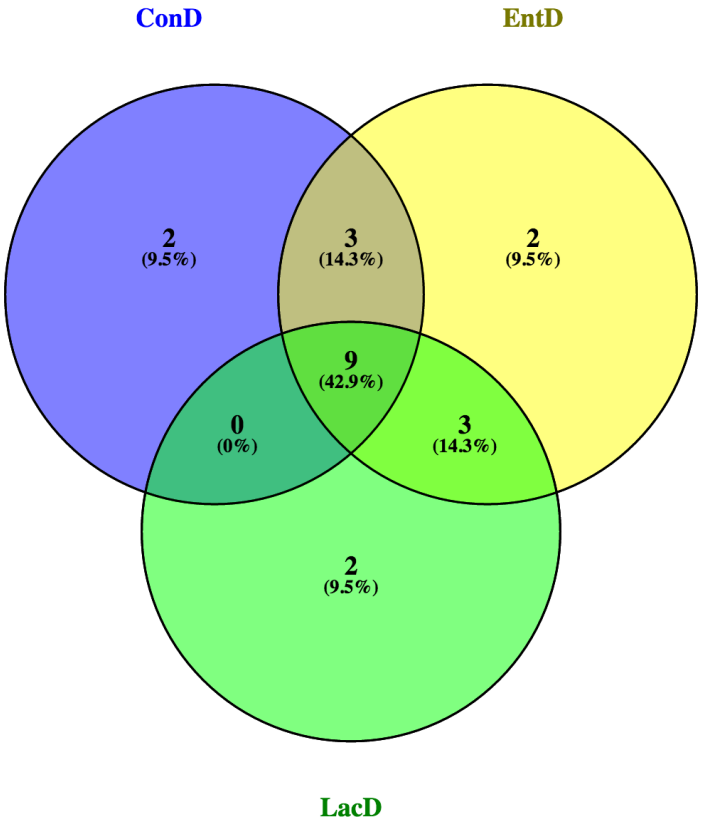

Figure 4.4: Differential features plots for Bacteroidia (order) (a), Clostridia (order) (b), *Lactococcus* sp. (c) and *Enterococcus* sp. (d), responsible for the main difference in pirarucu (*Arapaima gigas*) gut fed with probiotic *L. lactis* subsp. *lactis* mucosa (LacM) and digesta (LacD); fed with probiotic *E. faecium* mucosa (EntM) and digesta (EntD), and pirarucu without probiotic feeding mucosa (ConM) and digesta (ConD). Dashed line corresponds to median and full line corresponds to average between samples from the same treatment

A total of 9 shared OTUs (equating to 42.9% of the total OTUs abundance) were assigned as core microbiota in digesta samples, while 11 OTUs (50% of the total OTUs abundance) were detected in mucosa samples (Figure 4.5 and 4.6). The species *Bacillus coagulans* and the order Lactobacillales were exclusively present in the digesta of *Enterococcus*-fed fish, while the genera *Streptococcus* and *Alkalibacterium* were exclusively part of digesta of *Lactococcus*-fed fish. On the other hand, Bacteriodetes (family) were found only detected in the mucosa of *Enterococcus*-fed fish, and *Aquaspirillum putridiconchylium* was only detected in the mucosa of *Lactococcus*-fed fish.




| Con | Con x Lac | Con x Lac x Ent |
|---|---|---|
| Erysipelotrichaceae (family) | *Epulopiscium* sp. | Clostridiales (order) |
| *Streptococcus* sp. | | Clostridiaceae (family) |
| Lachnospiraceae (family) | **Con x Ent** | *Cetobacterium somerae* |
| *Robinsoniella peoriensis* | *Alkalibacterium* sp. | Bacteroidales (order) |
| *Staphylococcus epidermidis* | | *Enterococcus* sp. |
| *Coprococcus* sp. | **Lac x Ent** | *Turicibacter* sp. |
| | Enterococcaceae (family) | *SMB53* sp. |
| **Ent** | | *Bacteroides* sp. |
| Bacteroidetes (family) | | 02d06 sp. |
| | | Peptostreptococcaceae (family) |
| **Lac** | | |
| *Aquaspirillum putridiconchylium* | | Fusobacteriaceae (family) |

Figure 4.5: Venn diagram for unique and shared OTUs (species) for mucosa of pirarucu (*Arapaima gigas*) fed with probiotic *L. lactis* subsp. *lactis* mucosa (LacM) and digesta (LacD); fed with probiotic *E. faecium* mucosa (EntM) and digesta (EntD), and pirarucu without probiotic feeding mucosa (ConM) and digesta (ConD) showing 80% of samples in each compartment




| Con | Ent x Lac | Con x Ent x Lac |
|---|---|---|
| *Aquaspirillum putridiconchylium* | Bacteroidales (order) | Clostridiales (order) |
| *Bacteroides* sp. | *Enterococcus* sp. | Clostridiaceae (family) |
| **Ent** | Enterococcaceae (family) | 02d06 sp. |
| *Bacillus coagulans* | | *Cetobacterium somerae* |
| Lactobacillales (order) | **Con x Ent** | *Turicibacter* sp. |
| **Lac** | Fusobacteriaceae (family) | *SMB53* sp. |
| *Streptococcus* sp. | *Epulopiscium* sp. | Peptostreptococcaceae (family) |
| *Alkalibacterium* sp. | *Streptococcus* sp. | *Carnobacterium* sp. |
| | | *Lactococcus* sp. |

Figure 4.6: Venn diagram for unique and shared OTUs (species) for digesta of pirarucu (*Arapaima gigas*) fed with probiotic *L. lactis* subsp. *lactis* mucosa (LacM) and digesta (LacD); fed with probiotic *E. faecium* mucosa (EntM) and digesta (EntD), and pirarucu without probiotic feeding mucosa (ConM) and digesta (ConD) showing 80% of samples in each compartment

Significant differences were observed between all treatments, except between both probiotic-fed treatment digesta (LacD x EntD), for unweighted Unifrac, after PERMANOVA analysis (Table 4.3). However, few differences were detected in weighted Unifrac data between ConM x EntM; LacM x ConD; and EntM x ConD.

Table 4.3: PERMANOVA results of unweight and weighted UniFrac showing difference between categories of intestinal microbiota composition of Pirarucu (*A. gigas*): fed with probiotic *L. lactis* subsp. *lactis* mucosa (LacM) and digesta (LacD); fed with probiotic *E. faecium* mucosa (EntM) and digesta (EntD), and pirarucu without probiotic feeding mucosa (ConM) and digesta (ConD)

| | | Unweighted | | Weighted | |
|---|---|---|---|---|---|
| | | p-value | Pseudo-F/ t-value | p-value | Pseudo-F/ t-value |
| PERMANOVA | | 0.001 | 4.3519 | 0.007 | 3.3822 |
| Pair-wise test | LacD x EntD | 0.422 | 1.015 | 0.190 | 1.3459 |
| | LacD x ConM | 0.003 | 2.0519 | 0.243 | 1.1654 |
| | LacD x LacM | 0.004 | 2.8126 | 0.117 | 1.6588 |
| | LacD x EntM | 0.007 | 2.2321 | 0.100 | 1.9691 |
| | LacD x ConD | 0.026 | 1.7857 | 0.193 | 1.412 |
| | EntD x ConM | 0.003 | 2.2628 | 0.072 | 1.9764 |
| | EntD x LacM | 0.003 | 3.401 | 0.203 | 1.2514 |
| | EntD x EntM | 0.002 | 2.603 | 0.223 | 1.167 |
| | EntD x ConD | 0.006 | 1.881 | 0.014 | 2.6523 |
| | ConM x LacM | 0.003 | 1.8958 | 0.119 | 1.917 |
| | ConM x EntM | 0.011 | 1.7516 | 0.050 | 2.1234 |
| | ConM x ConD | 0.007 | 1.4904 | 0.601 | 0.66728 |
| | LacM x EntM | 0.018 | 1.5207 | 0.381 | 1.0502 |
| | LacM x ConD | 0.002 | 1.9322 | 0.017 | 2.7804 |
| | EntM x ConD | 0.003 | 1.7696 | 0.003 | 3.151 |

Consequently, Principal Coordinate Analysis (PCoA) plots reveal similar results. There was no clear separation among samples in unweighted Unifrac data (Figure 4.7a) apart from some samples from control groups (mucosa and digesta), which appear separate from the main cluster. However, samples from both probiotic-fed groups digesta (LacD and EntD) appear clustered together in weighted Unifrac (Figure 4.7b). The same was observed with samples from both probiotic-fed groups mucosa (LacM and EntM). Still, in both cases, there are samples that are not clustered, and appear closer to the groups without probiotic feeding (ConM and ConD).

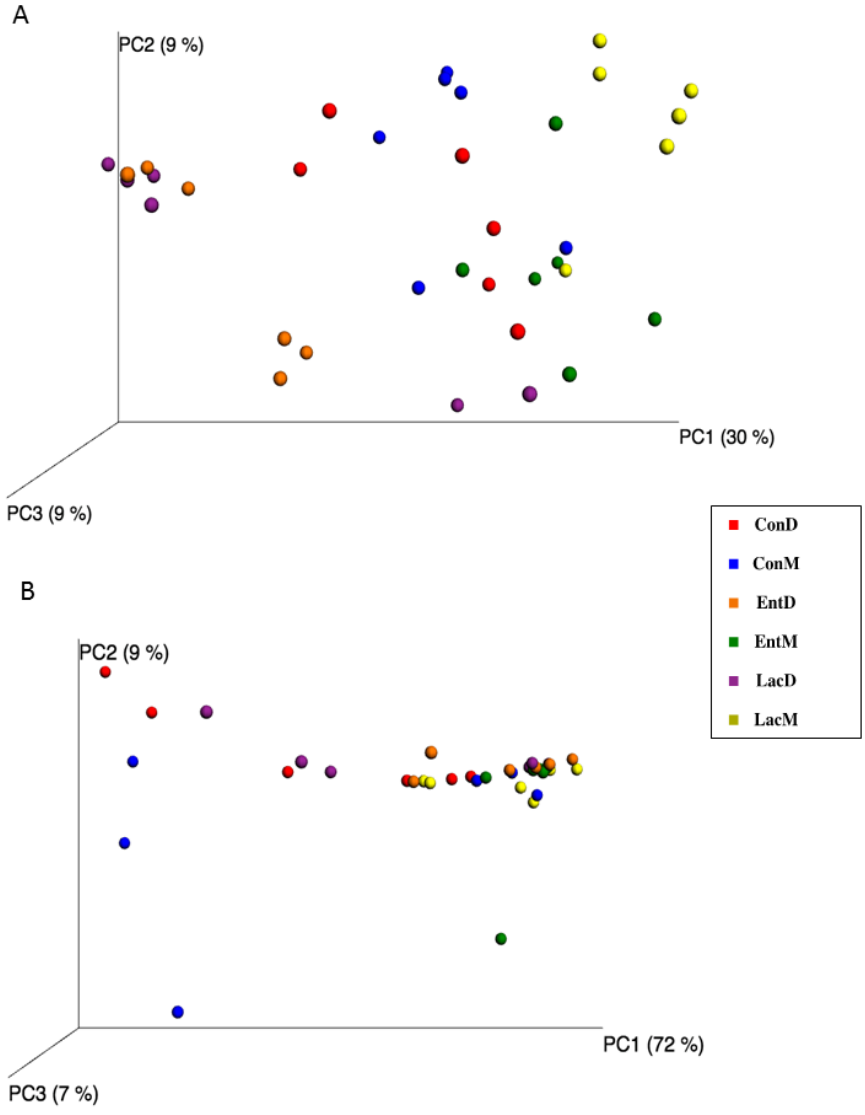



Figure 4.7: PCoA of Unweighted (A) and Weighted UniFrac (B) showing clustering of compartments for pirarucu (*A. gigas*) fed with probiotic *L. lactis* subsp. *lactis* mucosa (LacM) and digesta (LacD); fed with probiotic *E. faecium* mucosa (EntM) and digesta (EntD), and pirarucu without probiotic feeding mucosa (ConM) and digesta (ConD)

No difference in lamina-propria width, number of goblet cells and villi length was detected in histology analysis (Table 4.4). However, the number of epithelial eosinophils cells was higher in the group fed with *E. faecium* (Table 4.5). On the other hand, serum antimicrobial activity of the group fed with *L. lactis* subsp. *lactis* was higher against *Pseudomonas* sp. when compared with the other two treatments (Table 4.6).

Table 4.4: Red blood cells (RBC) concentration and percentage of differential white blood cells (WBC) in pirarucu (*A. gigas*) fed with diets containing probiotic *L. lactis* subsp. *lactis* (LAC); *E. faecium* (ENT), and no bacterial content (control)

| Treatment | Erythrocytes ($x10^6$) | Monocytes (%) | Neutrophils (%) | Eosinophils (%) | Basophils (%) |
|---|---|---|---|---|---|
| Control | 1.09±0.28 | 4.14±1.70 | 4.92±2.99 | 0.27±0.43[a] | 0.71±0.60 |
| LAC | 1.11±0.25 | 5.70±2.59 | 4.98±2.86 | 0.24±0.35[a] | 1.37±0.96 |
| ENT | 1.18±0.33 | 7.17±4.04 | 4.39±2.31 | 1.01±0.67[b] | 1.58±0.77 |

*Different superscripts indicate a significant difference ($p < 0.05$).

Table 4.5: Histological data from the posterior intestine of pirarucu (*A. gigas*) fed with diets containing *L. lactis* subsp. *lactis* (LAC); *E. faecium* (ENT), and no bacterial content (control)

| Treatment | Villi length | LP width | Goblet cells (mucins) | | | |
|---|---|---|---|---|---|---|
| | | | Acidic | Neutral | acidic+neutral | Total |
| Control | 360.59 ± 171.11 | 35.52 ± 23.93 | 5.16 ± 1.83 | 8.63 ± 3.05 | 7.48 ± 2.65 | 39.30 ± 8.77 |
| LAC | 406.50 ± 108.65 | 37.47 ± 10.05 | 4.08 ± 1.36 | 6.65 ± 2.22 | 18.13 ± 6.04 | 45.17 ± 19.61 |
| ENT | 387.76 ± 138.11 | 31.70 ± 4.66 | 8.19 ± 2.90 | 4.13 ± 1.46 | 15.94 ± 5.64 | 37.31 ± 16.36 |

Table 4.6: Immunological parameters of pirarucu (*A. gigas*) blood serum fed with diets containing probiotic *L. lactis* subsp. *lactis* (LAC), *E. faecium* (ENT), and and no bacterial content (Control)

| Treatment | Lysozyme (ul.ml$^{-1}$) | Antimicrobial (log x+1) | |
|---|---|---|---|
| | | *Pseudomonas* sp. | *Citrobacter freundii* |
| Control | 562.24± 369.22 | 0.15 ± 0.27[a] | 0.00 ± 0.00 |
| LAC | 540.76± 675.55 | 1.49 ± 0.93[b] | 0.86 ± 0.91 |
| ENT | 575.10± 481.88 | 0.00 ± 0.00[a] | 0.00 ± 0.00 |

*Different superscripts indicate a significant difference ($p < 0.05$)

## 4.5 Discussion

*Modulation of the gut microbiota*

This study assessed the *in vivo* modulation of two autochthonous strains in the gut microbiota of *Arapaima gigas* and their capacity to improve the fish gut health status. Although not commonly available, some studies have assessed the modulation of fish gut microbiota after probiotic feeding using HTS analysis, however, this is the first study to assess the intestinal gut microbiota after probiotic feeding in an Amazon basin native fish under captive conditions.

Firmicutes, Fusobacteria and Proteobacteria have been reported as dominant phyla in many fish species (Tarnecki et al., 2017). In the present study, the phyla Proteobacteria was not abundant when compared with Firmicutes and Fusobacteria that comprised more than 90% of the total abundance. However, this abundance was somewhat different from that reported in Chapter 3 where there was a higher abundance of Proteobacteria in juveniles and adult *Arapaima gigas*. This difference in abundance was not addressed by probiotic feeding in the present study, since there was no variance at phyla level between control and probiotic-fed groups. This may be because other external factors such as rearing system and host factors such as genetic diversity could influence microbial modulation in fish gut (Tarnecki et al., 2017).

At the genus level, a high percentage of the *Cetobacterium* suggests a key role of these bacteria in the gut of pirarucu. This genus commonly presents high abundance in intestine from many fresh water fish such as Nile tilapia (*Oreochromis niloticus*) (Adeoye et al., 2016); rainbow trout (*Oncorhynchus mykiss*) (Lyons et al., 2017); channel catfish (*Ictalurus punctatus*), largemouth bass (*Micropterus salmoides*) and bluegill (*Lepomis macrochirus*) (Larsen et al., 2014). Other authors also suggest that the species *Cetobacterium somerae* is able to inhibit the growth of potential bacterial pathogens (Sugita et al., 1996) and could produce high amounts of

vitamin B12 as well as peptide fermentation, highlighting its nutritional importance for carnivorous fish (Romero et al., 2014). Indeed, *Cetobacterium* species should be further studied to ascertain its role to freshwater fish intestinal tract, health and nutritional factors.

Alpha diversity parameters define the diversity of microorganisms in one sample. In the present study, Alpha diversity results suggest that a modulation has occurred in *A. gigas* gut microbiota in response to probiotic feeding. For instance, both Chao1 and number of observed species metrics estimate richness. They show the count of unique OTUs and the probability of new species to be presentin a sample, respectively. Thus, in the present study, the mucosal group community was richer in control fish when compared with *Enterococcus*-fed fish while the Phylogenetic Diversity (PD) was lower in both mucosa groups from probiotic fed fish. Standen et al. (2015) have also observed reduced richness (Chao1 and observed species) in tilapia (*Oreochromis niloticus*) fed with aprobiotic supplemented diet. This reduction could be due to competitive characteristic of probiotic bacteria that might be leading a decrease of other bacterial strains in the gut.

Indeed, while analysing HTS richness and diversity, both Alpha and beta diversity parameters should be taken into account to conclude a modulation in fish gut. For example, beta diversity parameters present the degree that each sample differs from one another based on species abundance. The PCoA plots represents this differences in a 3D spectrum. In unweighted Unifrac plot (represents the diversity of the samples without evaluating the abundance) there is a separation between treatments. Both probiotic mucosa samples appear to be similar between each other as well as both probiotic digesta clustered together (Figure 3 a) when compared to the control. However, in weighted Unifrac (represents the diversity counting the abundance in each sample) there is not a clear cluster in the diversity between treated fish. In the present study, the probiotic strains varied the diversity of the microbiota but did not modulate its relative abundance.

In this context, the linear discriminant analysis (LDA) and effect size (LefSE) results (and its differential features Figure 6) show the bacterial groups that drove the main differences between treatments. These results confirm PCoA results that there are differences between treatments. The absence of bacteria from the class Clostridia in the fish treated with probiotics suggests that the probiotic decreased the abundance of these strains in fish gut. Those are interesting results since the bacteria

from species *Clostridium botulinum* is considered a fish-borne zoonosis being able to affect humans by fish ingestion (Gauthier, 2015). LefSE results suggest that both probiotic bacteria are present in the digesta and they could be responsible for the differences in abundance of class Clostridia by their modulation and their presence in fish digesta content.

A high abundance of the strain *Enterococcus* sp. was observed in *A. gigas* gut of the treatments fed with this same bacterium and differed from the other treatments. The genus *Enterococcus* was also higher in tilapia (*O. niloticus*) fed with a commercial multi-strain probiotic, containing *Enterococcus faecium* (AquaStar® Growout) when compared with fish not fed with probiotic in diet (Standen et al. 2015) agreeing with the present study. The presence of the probiotic strains in the gut after being fed orally could indicate that they were able to survive the acidic conditions of the stomach and maintain activity to reach the intestinal environment. Merrifield at al. (2010) previously listed these characteristics as being important to characterise a bacterium as a probiotic bacterium, however, other parameters such as the health status of the fish should be analysed together to assure a probiotic effect.

*Physiological variables after probiotic feeding*

Blood analysis is an important tool for monitoring farmed animals because it can provide a routine response for physiological stress or disease response (Tavares-Dias et al. 2007). Hematological results in the present study are in agreement of normal values for farmed pirarucu (Tavares-Dias et al. 2007). In this case, eosinophil cells increase in fish blood after being fed with diet containing the bacteria *Enterococcus faecium*. Reite and Evensen (2006) have reported that eosinophilic cells act as defense in fish, however more studies should be done to support this same idea in fish fed with probiotic-supplemented diets.

The sustainable use of probiotics is related with their antimicrobial capacity (Lazado and Caipang 2014), thus the presence of antimicrobial-related molecules in fish serum is related with immunity. In the present study, *L. lactis* subsp. *lactis* increased the *in vitro* serum antimicrobial capacity of fish against *Pseudomonas* sp. when compared with the other treatments. This appear to be a positive result, however all the analyzed parameters should be put together to conclude a probiotic effect of the two tested strains.

## 4.6 Conclusion

The two autochthonous strains *E. faecium* and *L. lactis* subsp *lactis* were found to influence haemato-immunological parameters in *A. gigas*. They were also able to modulate intestinal microbiota in pirarucu and decrease the abundance of pathogenic bacteria in pirarucu gut after 21 days of feeding. These preliminary findings suggest both strains could populate the intestinal mucosa of *A. gigas.* Therefore, further investigation of the probiotics on pirarucu growth performance and health paraeters is warranted.

# Chapter 5:
# Growth performance and intestinal modulation of pirarucu *Arapaima gigas* fed with two different autochthonous probiotic bacteria

# Chapter 5: Growth performance and intestinal modulation of pirarucu *Arapaima gigas* fed with two different autochthonous probiotic bacteria

## 5.1 Introduction

As discussed in previous chapters, a potential probiotic candidate should have several important characteristics and their selection should follow strict stepwise procedure (see review of Banerjee and Ray, 2017). The efficacy of probiotic strains in relation to feeding duration in fish is limited and there is a large knowledge gap within this subject. The chapter 4 of this thesis have demonstrated that the isolated probiotic strains could modulate the gut microbiota, and provide some host benefits in terms of intestinal morphology over a short time period. However the present chapter seek to build on previous results and elucidate if the probiotic strains, and its intestinal modulatory effects, persist over a longer time period, and whether such benefits confer a growth promoting effect.

The relation between probiotic applications and fish growth performance is well known many aquatic animals (Banerjee and Ray, 2017). Marine fish such as olive flounder (*Paralichthys olivaceus*) fed with *Lactococcus lactis* (Nguyen et al., 2017) and Asian seabass (*Lates calcarifer*) fed with a probiotic mixture of four different strains (Lin et al., 2017) showed higher growth performance. Likewise, freshwater fish such as Mori (*Cirrhinus mrigala*) fed with commercial probiotic containing *Bacullis subtilis* (Ullah et al., 2018) and snakehead (*Channa striata*) fed with *Lactobacillus acidophilus* (Kibenge et al., 2004) presented improvements on growth parameters after being fed with these probiotics bacteria.

The relation of probiotic and fish growth performance is directly linked to probiotic feeding time. The duration of a probiotic intake can affect the colonization, mucosal biding, subsequent modulation of gut microbiota and immune responses in a host (Nayak, 2010). According Balcázar et al. (2006), the process of probiotic colonization happens after a long period of feeding because the multiplication rate is higher than its expulsion rate. This way the probiotic leads to the improvement of physiological parameters such as the specific growth rate (SGR).

For instance, differences in growth started being observed after 4 weeks of probiotic feeding in olive flounder (*Paralichthys olivaceus*) as well as feed conversion ratio and specific growth rate (Nguyen et al., 2017). These parameters were even higher after 8 weeks of prebiotic feeding. Munir et al. (2016) observed that snakehead (*Channa striata*), fed with *Lactobacillus acidophilus,* presented higher specific growth rate from 8 weeks to 16 weeks of feeding when compared with fish that received the control diet (non-supplemented), keeping higher than control until 24 weeks. In addition, weight gain (%) of grouper (*Epinephelus coioides*) fed with *Lactobacillus plantarum* was higher after two weeks of feeding when compared to control.

The giant fish of Amazon river, pirarucu, in south America already display excellent growth performance under different aquaculture systems cultivation (Bezerra et al., 2013), which could be improved with species specific probiotic intake. Thus, the objective of the present chapter was to evaluate the growth performance of pirarucu (*A. gigas*) after being fed with two different autochthonous strains *Lactococcus lactis* subsp. *lactis* and *Enterococcus faecium,* and their ability to modulate fish intestinal microbiota as well as immunological parameters, in a 42 days feeding trial.

## 5.2 Material and Methods

### Biological Material

The strains *Lactococcus lactis* subsp. *lactis* and *Enterococcus faecium* were previously isolated and identified (Chapter 3a). The strains were activated inoculating 1 colony in 9 ml MRS (Oxoid, UK) and incubated at 35 ˚C for 24-48 h.

One hundred and eighty *A. gigas* of 21.31 ± 4.03 g (weight ± SD) were removed from an excavated pond at the *Mar e Terra Ind. Com. de Pescados* fish farm (Rondônia state, Brazil) and randomly placed into nine tanks (1000 l) totalling 20 fish per tank. The tanks were placed in a flow-through system fed by a weir main pond. The water quality parameters were measured every two days and were normal for the pirarucu growth conditions. The water quality parameters were measured every two days to assure water quality. Dissolved oxygen (6.25 ± 1.02 $mg.l^{-1}$) and water temperature (27.05 ± 0.79°C) were measured using optical dissolved oxygen instrument (YSI, USA). The water pH (5.94 ± 0.25) and concentration of ammonia

(0.17 ± 0.07 $mg.ml^{-1}$), and nitrite (0.00 ± 0.00 $mg.ml^{-1}$) were measured using a kit for farmers to fresh water (Alfakit, SP, Brazil).

**Experimental design**

Fish were fed at 6% of biomass over four feeds per day (following the management protocols of the farm). The experiment lasted a total of 42 days and the treatments were as following: (1) fish fed commercial diet supplemented with *L. lactis* subps. *lactis*, (2) fish fed commercial diet supplemented with *E. faecium*, and (3) fish fed commercial diet without supplementation. An extruded commercial diet *Do Peixe Revolution Alevino 2 to 3 mm* (Douramix, Brazil) which contained 40% of crude protein and 11% of crude lipids was used during the experiment.

**Inoculation of probiotic bacteria**

The inoculation of both probiotic bacteria was performed as described in section 4.2.3 from Chapter 4. The probiotic concentration in the diets for this experiment was 1.56 ± 0.40 $x10^7$ $CFU.ml^{-1}$ and 7.94 ± 0.88 $x10^7$ $CFU.ml^{-1}$ for *L. lactis* subps. *lactis* and *E. faecium,* respectively.

**Sampling**

After the experiment period, 3 fish from each tank (n=9 per treatment) were sampled, anesthetized with 0.01% Eugenol (Vetec/Sigma-Aldrich), and terminated by destruction of the brain. The fish were externally washed with 70% ethanol , then, a cut was made on the abdomen using a sterile blade. Samples were collected as described in section 4.2.4 from chapter 4. Posterior intestine for histology analysis, and samples of mucosa and digesta for microbiological analysis using HTS as well as blood samples for differential leukocyte counts, total number of leukocytes, thrombocytes, and blood serum for immunological analysis were sampled as described in chapter 2, section 2.19. The methodology used for histology, HTS and hemato-immunological assays are described in chapter 2 (sections 2.21, 2.12 and 2.20, respectively).

**Quantitative real-time polymerase chain reaction (RT-qPCR)**

**Primer efficiency**

The resultant nucleotide sequences from the identification of both bacterial strains (section 2.10 from Chapter 2) were used for primer design using Primer3web

software (http://primer3.ut.ee/). All primers were synthetized by Eurofins MWG (Ebersberg, Germany). The primers used are listed in Table 5.1.

Table 5.1: Specific primers used quantitation of *Lactococcus* sp. (Lac) and *Enterococcus* sp. (Ent) using RT-qPCR.

| **Name** | **Sequence** | **Melting T°C** | ***E**** | **PS*** | **Accession number** |
|---|---|---|---|---|---|
| Lac (fwd) | 5'-ATCACCGGCAGTCTC GTTAGA-3' | 60.95 | 3.46 | 80 | MF990372.1 |
| Lac (rev) | 5'-GGTTAAGTCCCGCAACGA-3' | 57.32 | | | |
| Ent (fwd) | 5'-TCCATGTGTAGCGGTGAAATG-3' | 58.56 | 3.29 | 85 | KU359788.1 |
| Ent (rev) | 5'-GCCTCAGCGTCAGTTACAG-3' | 58.26 | | | |

*E= efficiency value; PS= product size.

For primer optimization, PCR for each set of primers were determined using serial dilutions of cDNA ($n$ = 3). A reaction of 20 ul where: 10 ul of SYBR® Green Supermix (Bio- Rad, CA, USA), 6.8 ul of molecular grade water (DEPC-treated water Ambion), 1.2 ul of each primer (Forward + Reverse at 0.3 uM) and 2 ul of the DNA of each dilution of each cDNA was performed. The reaction was plated in triplicate in a 48-well microplate and run the standard thermal profile (10 min at 95 °C and then 40 cycles of 15 s at 95 °C, 60 s at 60 °C) in StepOne Plus™ Real-time PCR thermal cycler (Applied Biosystems, Life Technologies). The point, which the fluorescence rises appreciably above the background fluorescence, was determined manually for each run and defined as the threshold cycle (Ct) and resulting plots of Ct versus the logarithmic cDNA input, using the equation $E$ (PCR efficiency) = 10x(-1/slope). (Rasmussen, 2001).

**Standard curve**

To perform DNA quantitation in intestine of pirarucu, pure colonies of the two probiotic strains *E. faecium* and *L. lactis* subsp. *lactis* were inoculated in MRS broth (Oxoid, UK) and incubated for 24h at 37 °C. The strains where then diluted 1:10 of PBS and plated onto MRS agar (Oxoid, UK) plates with 1% of aniline blue 1% (v/v) for quantification (CFU.ml$^{-1}$). The DNA was extracted from each dilution using the same protocol described in section 2.6 from Chapter 2.

The extracted DNA was used as template to perform the standard curves. A reaction of 7.5 ul where: 3.5 ul of SYBR® Green Supermix (Bio- Rad, CA, USA), 1.3 ul of molecular grade water, 0.45 ul of each primer (Forward + Reverse at 0.3 uM) and 2 ul of the DNA extracted from each dilution ($10^{-3}$ to $10^{-7}$ for *Enterococcus* sp. and $10^{-1}$ to $10^{-6}$ for *Lacotoccus* sp.) was prepared. The standard curve was constructed by plating the reaction in triplicate in a 384-well microplate and run the standard thermal profile (same as above) in QuantStudio® 12K Flex Real-Time PCR system (Applied Biosystems, Life Technologies). This protocol was repeated until $R^2$ of each curved was >0.97 (See supplementary Figure S1).

**pPCR quantitation**

The extracted DNA from mucosa tissue from two fish per tank (n=6 per treatment) was used as cDNA template to perform quantitation of *E. faecium* and *L. lactis* subps. *lactis* in intestine of *A. gigas*. The PCR reaction of 7.5 ul (as described in the *standard curve* section) was performed in duplicate for all samples and plated in a 384-well microplate and the standard thermal profile was used in QuantStudio® 12K Flex Real-Time PCR system.

The quantification of each bacterial species in the fish intestinal mucosal sample was calculated based on the equation of standard curve as previously mentioned and reported as $CFU.ml^{-1}$.

## 5.3 Statistical analysis

Statistical analyses were conducted as described in section 2.24 from chapter 2.

## 5.4 Results

After trimming and removal of low quality reads, HTS of bacterial 16S rRNA V1-V2 regions from the Ion Torrent® PGM resulted in 1,486,554 reads. *Streptophyta* reads were considered a contaminant from chloroplasts in feed materials and were then removed and filtered (at 0.005%) (Gajardo et al. 2016) resulting in 1,478,053 reads, of which 258,177 and 224,584 was assigned for the groups control digesta (ConD) and control mucosa (ConM); 258,119 and 257,276 for *Lactococcus*-fed fish digesta (LacD) and mucosa (LacM); 241,035 and 238,862 for *Enterococcus*-

fed fish digesta (EntD) and mucosa (EntM). The total number of operational taxonomic units (OTUs) assigned from all groups was 253 observations.

Intestinal microbiota was dominated by phyla Fusobacteria and Firmicutes (90% to 95% comprised together) in all groups and no statistical difference observed across the treatments (Table 5.2).

Table 5.2: Percentage of abundance (± standard deviation) of intestinal microbiota at phylum level of pirarucu (*A. gigas*) fed for 42 days with probiotic *L. lactis* subsp. *lactis* mucosa (LacM) and digesta (LacD); fed with probiotic *E. faecium* (EntM) and digesta (EntD), and pirarucu without probiotic feeding mucosa (ConM), and digesta (ConD)

| | **Other*** | **Bacteroidetes** | **Firmicutes** | **Fusobacteria** | **Proteobacteria** |
|---|---|---|---|---|---|
| ConD | 1.64±3.86 | 7.33±8.45 | 12.08±11.18 | 77.04±14.59 | 1.9±1.51 |
| EntD | 0.05±0.06 | 2.51±3.09 | 4.57±2.7 | 91.51±6.75 | 1.36±2.48 |
| LacD | 0.14±0.17 | 6.77±10.26 | 11.23±11.62 | 80.34±20.87 | 1.53±1.29 |
| ConM | 0.66±0.73 | 2.79±1.77 | 9.22±4.05 | 84.86±5.22 | 2.46±1.3 |
| EntM | 0.13±0.11 | 3.51±2.54 | 11.89±7.99 | 83.39±7.61 | 1.08±0.77 |
| LacM | 0.51±0.57 | 4.78±3.7 | 12.6±11.68 | 80.71±11.9 | 1.4±1.08 |

*Other: assigns the reads lower than 0.01 (Actinobacteria, OD1 and Verrucomicrobia) and non-identified phylum (Other)

At genus level, sequences assigned as *Enterococcus* sp. were detected in all treatments (Figure 5.1a) with the exception of control fish digesta (ConD). Higher abundance of *Enterococcus* sp. was detected in fish intestinal digesta samples from both treatments fed with one of the probiotics (LacD and EntD) when compared with control ($p$=0.022 and $p$=0.013 for *Lactococcus*-fed and *Enterococcus*-fed, respectively). Whilst, the abundance of *Enterococcus* sp. in fish intestinal mucosa from fish *Enterococcus*-fed treatment was higher when compared with *Lactococcus*-fed fish ($p$=0.015). On the other hand, *Lactococcus* sp. was not detected in fish under any treatment.

The OTU assigned as *Cetobacterium* genus were the most abundant in all treatments for both mucosa and digesta samples (Figure 5.1b), raging between 77% and 91% of total reads. The order Bacteroidales and genus *02d06* (Clostridiaceae family) represent the second and third most abundant for control fish mucosa (2.66% and 1.81%), and digesta (6.40% and 4.45%), respectively. Similar results were found in *Lactocccus*-fed fish intestine, where Bacteroidales (order) and genus *02d06* are the most representative after *Cetobacterium* in digesta (5.17% and 4.11%, respectively), however Lactobacillaceae family and order Bacteroidales were found in mucosa samples (5.60% and 3.89%, respectively). On the other hand, *Enterococcus*-fed fish

intestines show order Bacteroidales and Lactobacillaceae family as second and third most abundant in mucosa (3.31% and 2.26%, respectively), while digesta samples comprised the orders Bacteroidales and Clostridiales (2.39% and 1.26%, respectively).

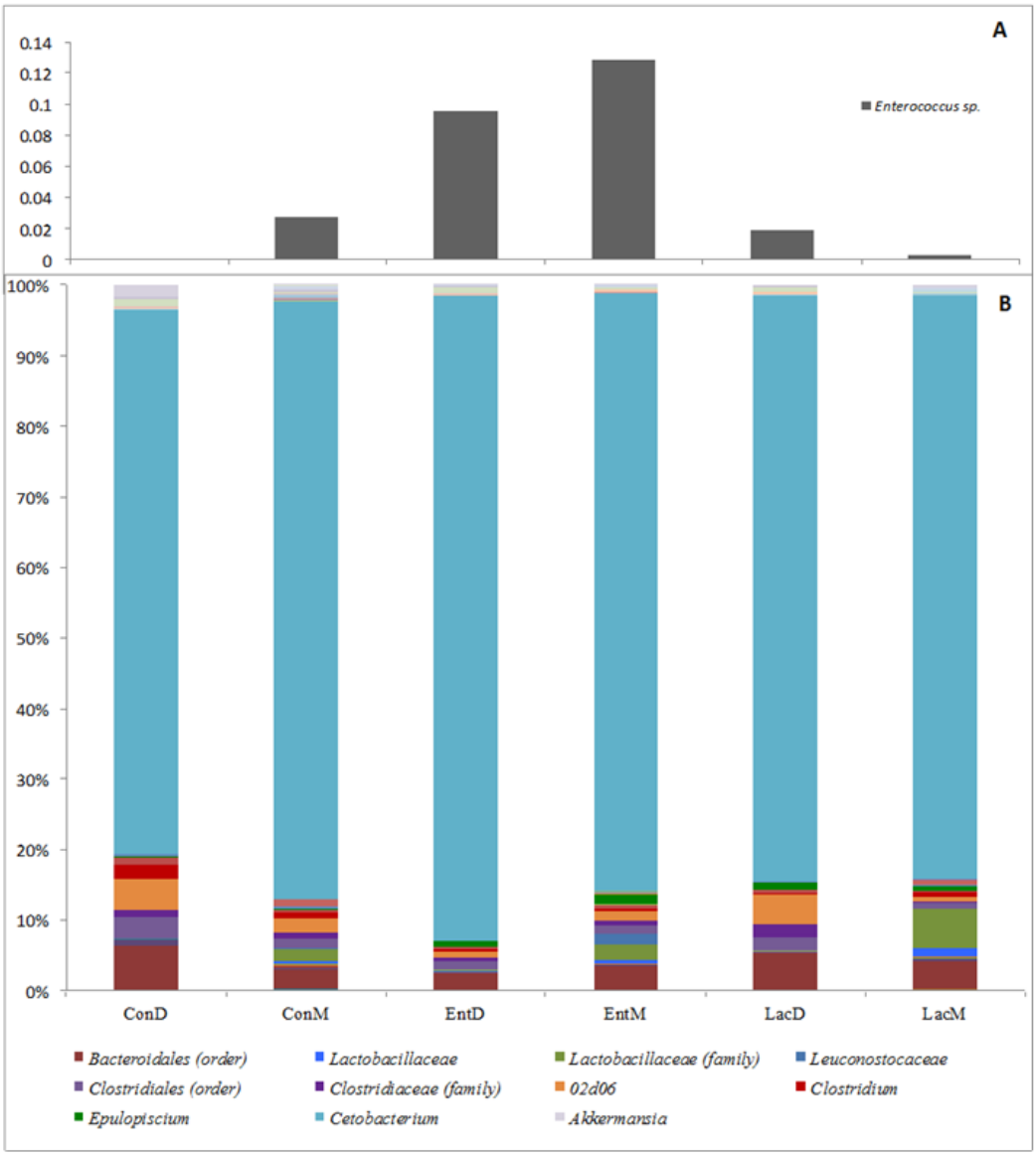



Figure 5.1: *Enterococcus* sp. composition average (%) (A), and total taxonomic composition average (%) of the at genus level (B). The plots represent the abundance in each category of pirarucu (*A. gigas*) after 42 days feeding with probiotic *L. lactis* subsp. *lactis* mucosa (LacM) and digesta (LacD); fed with probiotic *E. faecium* mucosa (EntM) and digesta (EntD); and pirarucu without probiotic feeding mucosa (ConM) and digesta (ConD)

The OTUs distribution assigned at the genus level were different in mucosal samples according Linear discriminant analysis (LDA) effect size (LEfSe) results (Figure 5.2a and b). The class Bacilli was significant different in the intestines of fish fed with *Lactococcus lactis* subsp. *lactis*. *Pseudomonas* genus also appears as abundant in fish under this same treatment. On the other hand, Actinobacteria phylum and Alphaproteobacteria class were statistically different in mucosa from non-probiotic fed fish (control). It is important to highlight the presence of *Enterococcus* genus was significantly higher in mucosa from fish fed with *Enterococcus faecium* when compared with the other treatments. Differential features show the main difference found across the treatments (Figure S5.1).

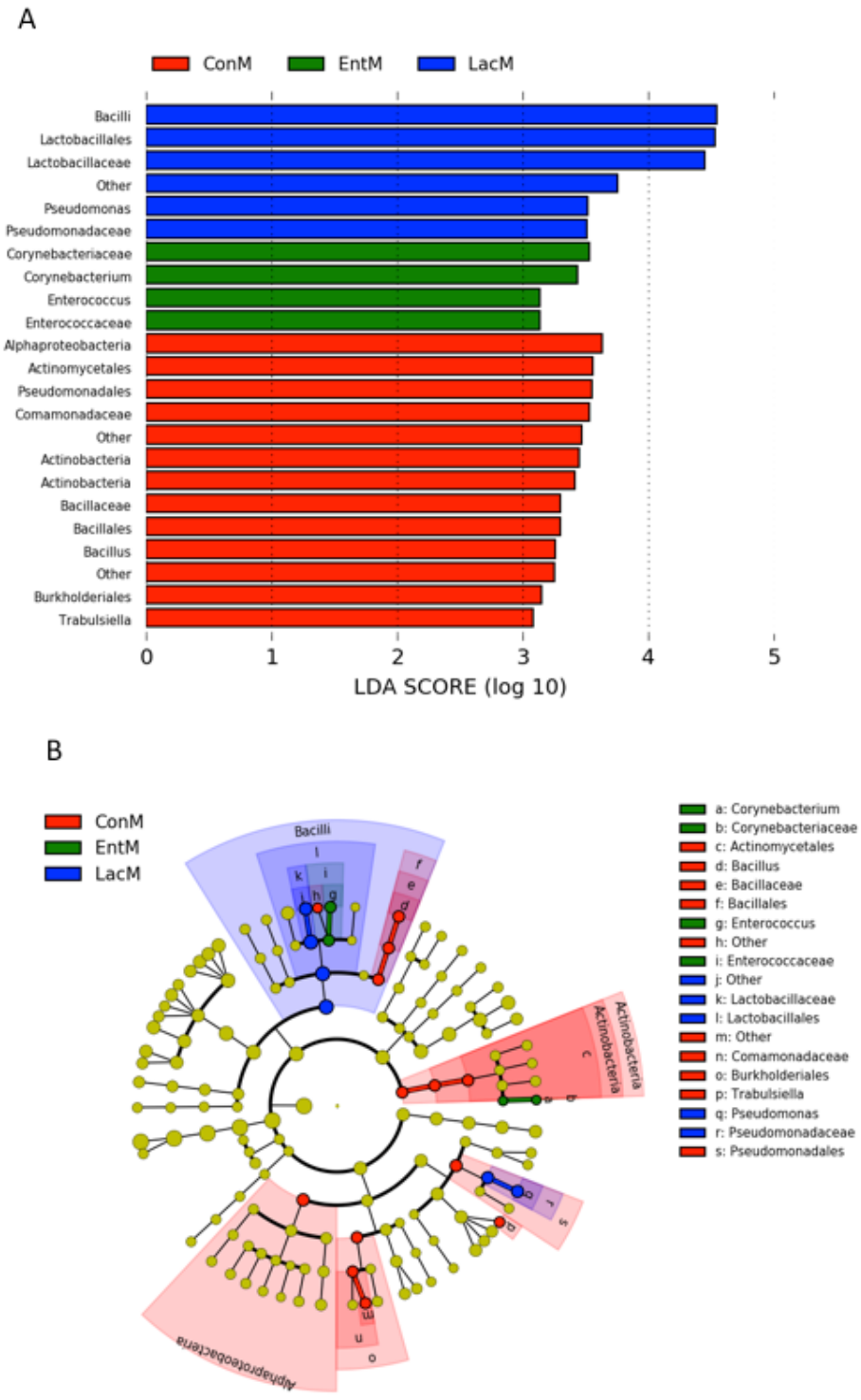



Figure 5.2: LDA (A) and cladogram plot LEfSe (B) showing the OTUs distribution around the circle after 42 days of the treatments: Control digesta (ConD) and mucosa (ConM); *L. lactis* subsp. *lactis* digesta (LacD) and mucosa (LacM); and *E. faecium* digesta (EntD). Colours represent the most abundant bacteria in each category (yellow = no difference detected). *No difference was detected digesta samples

The Alpha diversity parameters show no difference in Chao1 index and number of observed species; however, phylogenetic diversity (PD) in mucosa appears to be different from digesta in all treatments (Table 5.3 and Figure 5.3). Likewise, Principal Coordinate Analysis (PCoA) plots displays all digesta samples clustered together while separated from mucosa samples (Figure 5.4). Beta diversity parameters revels significant differences mainly for unweigthed Unifrac after PERMANOVA analysis (Table 5.4). Weighted Unifrac results did not present statistical differences.

Table 5.3: Alpha parameters results (Chao1 index, Observed Species, and PD Whole Tree) of intestinal microbiota composition of pirarucu (*A. gigas*) after 42 days feeding with probiotic *L. lactis* subsp. *lactis* mucosa (LacM) and digesta (LacD); fed with probiotic *E. faecium* (EntM) and digesta (EntD), and pirarucu without probiotic feeding mucosa (ConM) and digesta (ConD)

| Treatment | Chao1 | Observed species | Phylogenetic diversity (PD) |
|---|---|---|---|
| ConD | 160.56±10.15$^{a}$ | 140.75±10.12$^{a}$ | 3.64±0.54$^{a}$ |
| LacD | 155.82±18.9$^{a}$ | 130.85±25.32$^{a}$ | 3.67±0.37$^{a}$ |
| EntD | 165.21±15.49$^{a}$ | 136.97±23.05$^{a}$ | 3.7±0.47$^{a}$ |
| ConM | 178.48±16.63$^{ab}$ | 155.32±19.65$^{ab}$ | 5.17±0.52$^{b}$ |
| LacM | 196.29±10.15$^{abc}$ | 162.38±13.67$^{b}$ | 4.86±0.36$^{b}$ |
| EntM | 202.77±9.73$^{bc}$ | 178.42±7.99$^{b}$ | 5.02±0.38$^{b}$ |

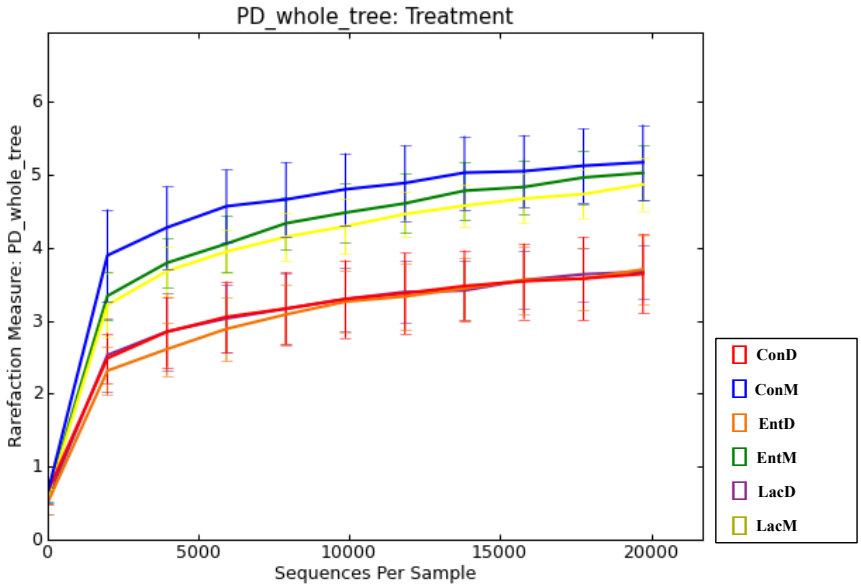



Figure 5.3: Rarefaction curve for Phylogenetic Diversity (PD) for pirarucu (*A. gigas*) fed with probiotic *L. lactis* subsp. *lactis* mucosa (LacM) and digesta (LacD); fed with probiotic *E. faecium* mucosa (EntM) and digesta (EntD); and pirarucu without probiotic feeding mucosa (ConM) and digesta (ConD)

Table 5.4: PERMANOVA results of unweight and weighted UniFrac showing difference between categories of intestinal microbiota composition of Pirarucu (*A. gigas*): after 42 days fed with probiotic *L. lactis* subsp. *lactis* mucosa (LacM) and digesta (LacD); fed with probiotic *E. faecium* mucosa (EntM) and digesta (EntD), and pirarucu without probiotic feeding mucosa (ConM) and digesta (ConD)

| | | **Unweighted** | | **Weighted** | |
|---|---|---|---|---|---|
| | | **p-value** | **Pseudo-F/ t-value** | **p-value** | **Pseudo-F/ t-value** |
| PERMANOVA | | 0.001 | 2.8245 | 0.164 | 1.3328 |
| Pair-wise test | LacM x ConM | 0.359 | 1.0499 | 0.674 | 0.83934 |
| | LacM x EntD | 0.013 | 1.6281 | 0.06 | 1.4062 |
| | LacM x LacD | 0.009 | 1.7412 | 0.352 | 1.0283 |
| | LacM x ConD | 0.008 | 1.8118 | 0.176 | 1.2771 |
| | LacM x EntM | 0.735 | 0.87265 | 0.977 | 0.55243 |
| | ConM x EntD | 0.003 | 2.1903 | 0.08 | 1.4081 |
| | ConM x LacD | 0.006 | 2.2105 | 0.397 | 1.075 |
| | ConM x ConD | 0.005 | 2.2616 | 0.121 | 1.4035 |
| | ConM x EntM | 0.061 | 1.3139 | 0.423 | 0.96323 |
| | EntD x LacD | 0.732 | 0.82668 | 0.359 | 1.1452 |
| | EntD x ConD | 0.283 | 1.1113 | 0.057 | 1.7904 |
| | EntD x EntM | 0.007 | 1.8988 | 0.098 | 1.4551 |
| | LacD x ConD | 0.246 | 1.1229 | 0.825 | 0.48964 |
| | LacD x EntM | 0.007 | 1.9782 | 0.402 | 1.0744 |
| | ConD x EntM | 0.007 | 2.1021 | 0.1 | 1.4175 |

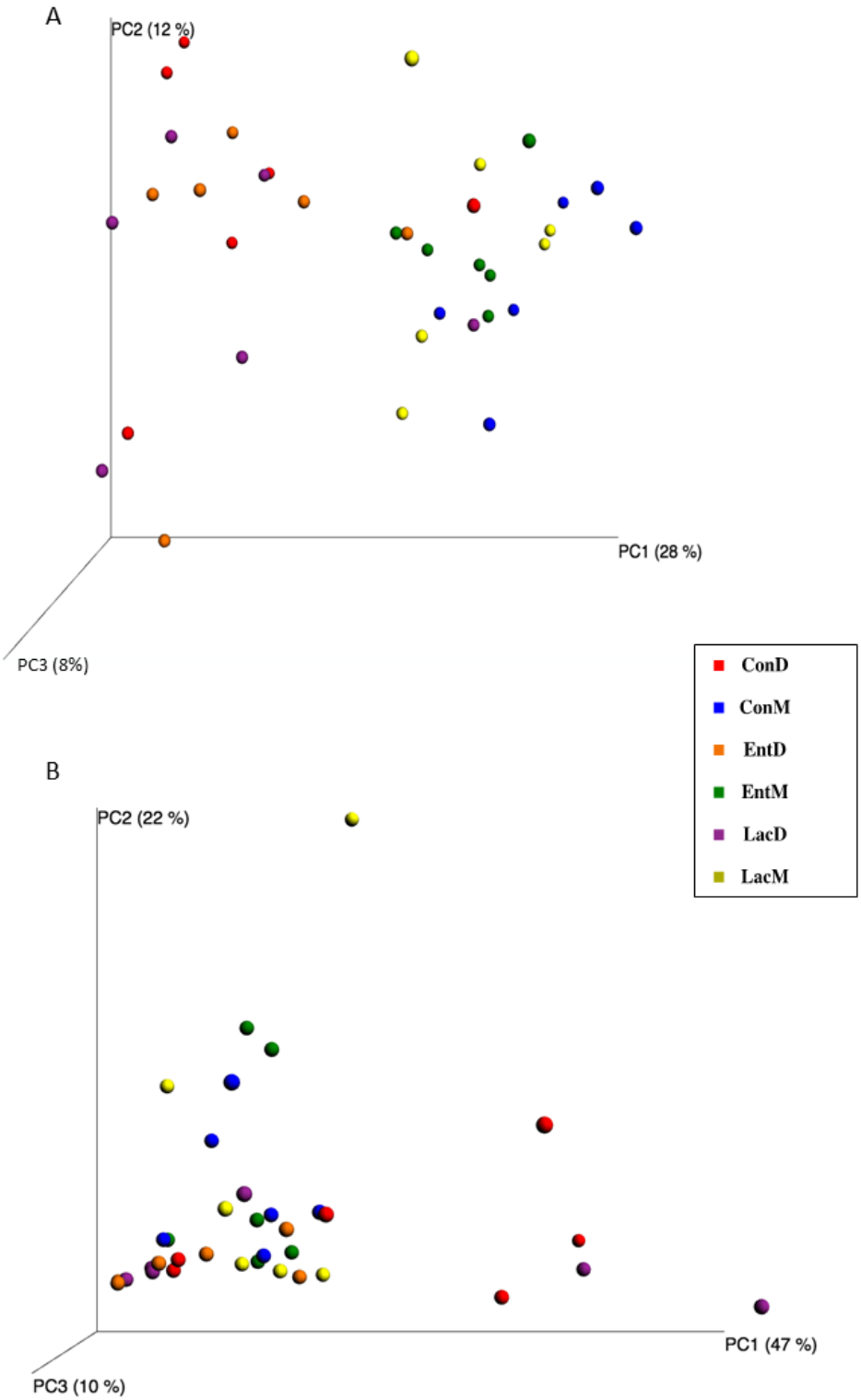



Figure 5.4: PCoA of Unweighted (A) and Weighted UniFrac (B) showing clustering of compartments for pirarucu (*A. gigas*) after 42 feeding with probiotic *L. lactis* subsp. *lactis* mucosa (LacM) and digesta (LacD); fed with probiotic *E. faecium* mucosa (EntM) and digesta (EntD), and pirarucu without probiotic feeding mucosa (ConM) and digesta (ConD)

Figure 5.5 reveals the shared and unique OTUs across the treatments. In digesta samples, *Parabacteroides* sp., *Clostridium difficile*, Bacteroidetes (phylum), Bacteroidaceae (family), and a non-identified bacterium appeared as unique components of the intestinal microbiomes of the non- fed fish (ConD). The OTUs identified as Lachnospiraceae (family), *Enterococcus* sp., and *Pseudomonas* sp. were the representative of *Enterococcus*-fed fish (EntD), while *Aquaspirillum* represented unique OTUs for *Lactococcus*-fed individuals (LacD). In mucosa samples, the unique OTUs for non-supplemented fish (ConM) were: *Methylosinus* sp., *Enterococcus*, Methylobacteriaceae (family), *Microbacterium* sp., *Enterobacter*, and *Stenotrophomonas*. The OTUs *Janthinobacterium lividum*, *Enterococcus* sp., Leuconostocaceae (family) and *Coprococcus* sp. appeared only in *Enterococcus*-fed fish (EntM). However, Aeromonadaceae (family) and *Micrococcus luteus* were identified only in *Lactococcus*-fed (LacM) fish mucosa.

Quantitative real-time polymerase chain reaction revealed that the concentration of *Enterococcus* sp. DNA present in mucosa was higher ($p$=0.042) in fish fed with *E. faecium* strain (9.61 Log CFU.ml$^{-1}$) when compared to fish fed with non-supplemented diet (7.169 Log CFU.ml$^{-1}$). Also, the concentration of *Lactococcus* sp. DNA was higher ($p$=0.017) in mucosa of fish fed with *E. faecium* strain when compared to the other two groups (Figure 5.6a and b).

Growth performance parameters for pirarucu fed with probiotic bacteria for 42 days are shown in Table 5.5. Fish fed with *Lactococcus lactis* subsp. *lactis* (LAC) presented higher percentage increase (%I) for weight, and specific growth rate (SGR) when compared with fish that received the non-probiotic diet. However, no difference was detected for proximate carcass composition across the treatments (Table 5.6) as well as the histological parameters (Table 5.7).

Haemato-immunological parameters are displayed in Table 5.8. A higher percentage of monocytes was observed in blood from pirarucu fed with *Lactococcus lactis* subsp. *lactis* (LAC) when compared with both other treatments, however, no difference was observed in antimicrobial activity in fish under the treatment tested in this experiment.

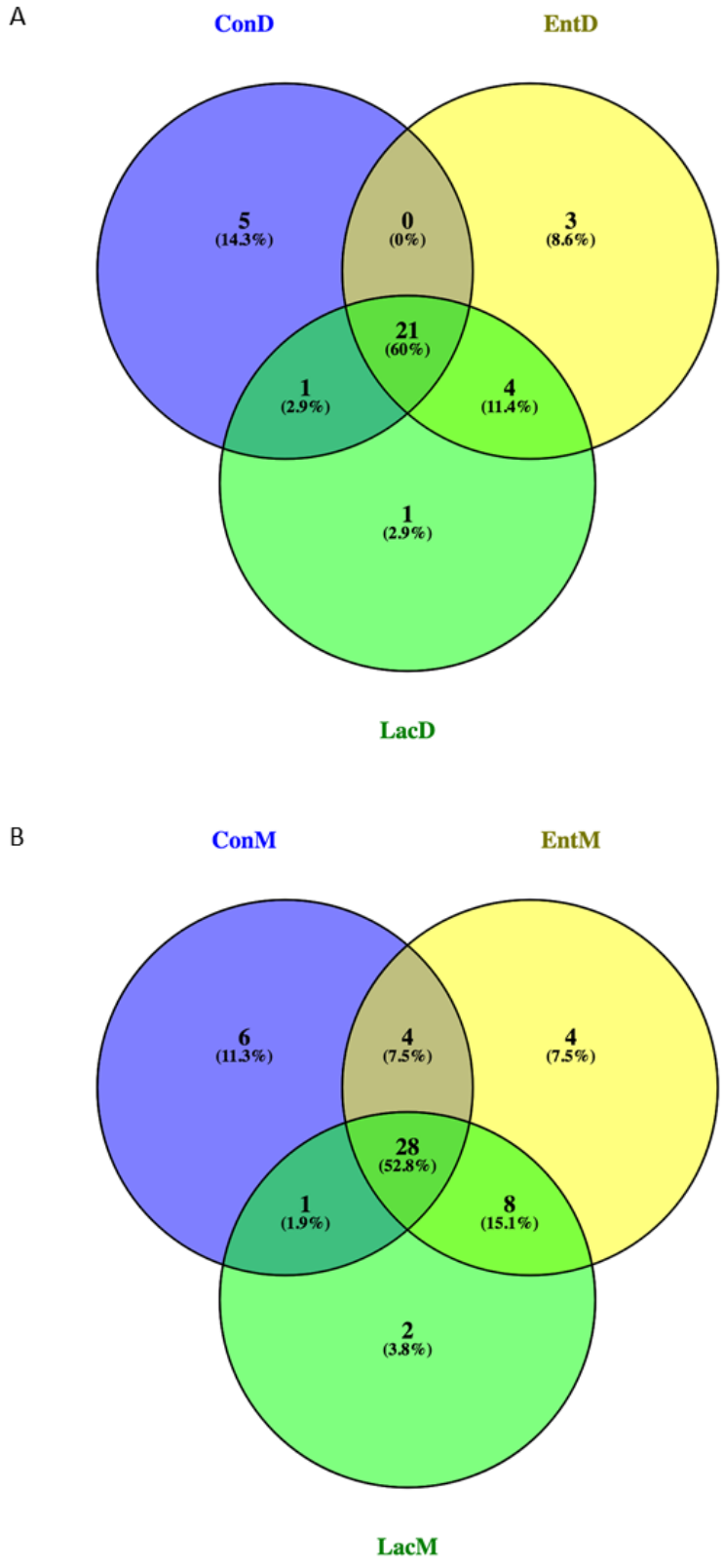



Figure 5.5: Venn diagram for unique and shared OTUs (species) for Digesta (A) and Mucosa (B) of pirarucu (*Arapaima gigas*) after 42 days feeding with probiotic *L. lactis* subsp. *lactis* mucosa (LacM) and digesta (LacD); fed with probiotic *E. faecium* mucosa (EntM) and digesta (EntD), and pirarucu without probiotic feeding mucosa (ConM) and digesta (ConD) showing 80% of samples in each compartment

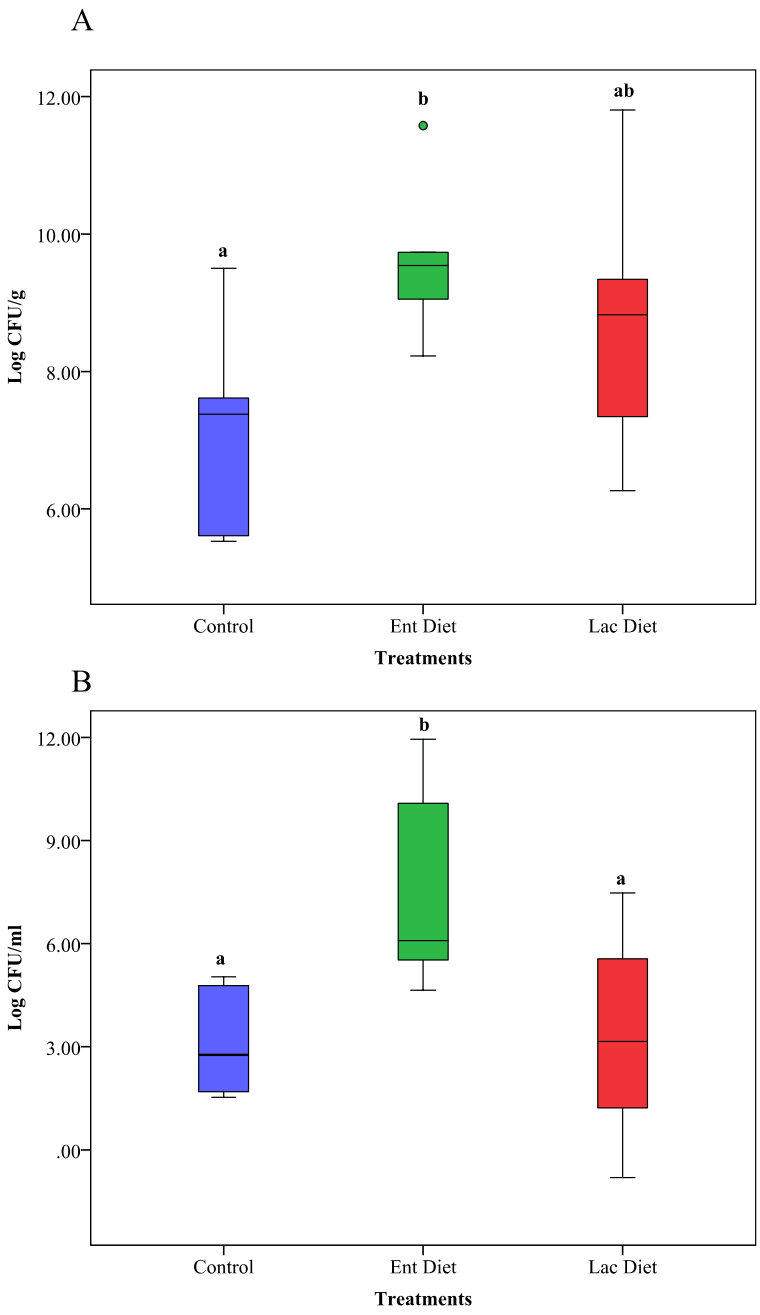



Figure 5.6: Quantitative real-time polymerase chain reaction (RT-qPCR) for intestinal mucosa DNA concentration of *Lactococcus* sp. (B) and *Enterococcus* sp. (A) of pirarucu (*Arapaima gigas*) after 42 days feeding with probiotic *L. lactis* subsp. *lactis* (Lac Diet); fed with probiotic *E. faecium* (Ent Diet), and pirarucu without probiotic aministration (Control). [a,b] Different subscripts indicate a significant different ($p<0.05$)

Table 5.5: Growth performance parameters (average ± standard deviation) for pirarucu, *A. gigas*, after 42 days feeding with *Lactococcus lactis* subsp. *Lactis* (LAC); *Enterococcus faecium* (ENT); and non-supplemented diet (Control).

| Parameter | Control | LAC | ENT |
|---|---|---|---|
| Initial Weight (g) | 21.75±3.21 | 20.63±3.44 | 21.57±3.8 |
| Final Weight (g) | 91.99±10.87 | 96.37±16.25 | 94.78±15.08 |
| Final Length (cm) | 22.54±0.59 | 22.63±1.34 | 22.59±1.09 |
| Net Weight Gain (g) | 70.24±7.85 | 75.73±12.9 | 73.22±11.3 |
| FCR | 3.22±0.17 | 3.2±0.06 | 3.25±0.07 |
| PER (g $g^{-1}$) | 0.81±0.08 | 0.77±0.13 | 0.76±0.21 |
| SGR (% $day^{-1}$) | $3.44±0.12^{a}$ | $3.67±0.09^{b}$ | $3.53±0.05^{ab}$ |
| % I | $324.31±21.32^{a}$ | $367.32±17.53^{b}$ | $340.33±10.02^{ab}$ |
| Condition factor k | 0.80±0.03 | 0.83±0.01 | 0.82±0.01 |

[a,b] Different subscripts indicate a significant different ($p<0.05$). FCR = Feed conversion factor; PER = Protein efficiency ratio; SGR = specific growth rate; %I = percentage of increase

Table 5.6: Proximate carcass composition (%) of pirarucu, *A. gigas*, after 42 days feeding with *Lactococcus lactis* subsp. *lactis* (LAC); *Enterococcus faecium* (ENT); and non-supplemented diet (Control).

| Treat | Moisture | Ash* | Lipids* | Protein* |
|---|---|---|---|---|
| Control | 83.53±0.54 | 21.38±0.54 | 6.03±0.58 | 62.31±0.83 |
| ENT | 83.51±0.47 | 21.41±0.54 | 7.57±0.55 | 61.74±0.72 |
| LAC | 84.20±0.54 | 21.51±0.57 | 6.68±0.58 | 62.90±0.97 |

*Values for ash, lipids and protein are reported as percentage of dry matter

Table 5.7: Histological data from posterior intestine of pirarucu, *A. gigas*, after 42 days feeding with *Lactococcus lactis* subsp. *lactis* (LAC); *Enterococcus faecium* (ENT); and non-supplemented diet (Control)

| | **Villi length** | **LP width** | **Goblet cells (mucins)** | | |
|---|---|---|---|---|---|
| | | | **Acidic** | **Acidic+neutral** | **Total** |
| Control | 492.56±112.43 | 28.87±2.33 | 8.39±2.29 | 2.39±2.17 | 10.78±3.98 |
| ENT | 478.88±27.24 | 27.6±6.36 | 6.68±1.38 | 5.83±2.24 | 14.88±1.68 |
| LAC | 469.68±76.1 | 30.46±4.37 | 5.99±3.54 | 6.79±2.33 | 12.78±1.56 |

Table 5.8: Haemato-immunological data from pirarucu, *A. gigas*, after 42 days feeding with *Lactococcus lactis* subsp. *lactis* (LAC); *Enterococcus faecium* (ENT); and non-supplemented diet (Control)

| **Treatment** | **RBC** | **WBC** | | | **Antimicrobial (log x+1)** | |
|---|---|---|---|---|---|---|
| | **Erythrocytes (x10$^5$)** | **Thrombocytes (%)** | **Monocytes (%)** | **Neutrophils (%)** | *Pseudomonas* sp. | *Citrobacter freundii* |
| Control | 7.88±3.95 | 9.69±5.63 | 8.87±6.24$^a$ | 4.82±4.03 | 2.19±0.83 | 0.0±0.0 |
| ENT | 9.77±5.59 | 9.68±4.03 | 9.97±5.54$^a$ | 7.6±5.36 | 2.43±0.54 | 0.0±0.0 |
| LAC | 12.2±5.17 | 5.16±4.3 | 15.23±5.57$^b$ | 7.61±4.18 | 1.79±1.16 | 0.0±0.0 |

$^{a,b}$ Different subscripts indicate a significant different ($p$<0.05).

## 5.5 Discussion

The currently chapter provides novel information on the efficacy of the two autochthonous probiotics on the growth performance of *A. gigas*, and their ability to modulate microbiota over a longer time period (42 days) than previously described in chapter 4. In addition, the present chapter data regarding the modulation of gut microbiota using the new technology high-throughput sequencing, which revealed a detailed map on the gut microbiome after 42 days of feeding with two different autochthonous probiotic bacteria.

*Modulation in gut microbiota*

The phylum Proteobacteria, Fusobacteria and Firmicutes were described as part of gut core-microbiota in many different fish species (Ghanbari et al., 2015). Fusobacteria and Firmicutes phyla represent almost the total of reads (90% to 95% comprised together) within treatments of the present study, however, Proteobacteria phyla do not appear to be abundant in *A. gigas* intestine under the current study conditions.

One exemplar of the Fusobacteria phylum, the *Cetobacterium* is a notorious representative of the fish intestines and is known for its vitamin B12 production (Tsuchiya et al., 2008; Wu et al., 2010). In the present study this genus appears as the most abundant in all treatments and may have an important role in pirarucu intestine metabolism. Indeed, the species *Cetobacterium somerae* have being recently described as presenting high abundance in freshwater fish such as Nile tilapia (*Oreochromis niloticus*), rainbow trout (*Oncorhynchus mykiss*) and channel catfish (*Ictalurus punctatus*) (Adeoye et al., 2016; Larsen et al., 2014; Lyons et al., 2017). Other authors reported that this species has ability to inhibit pathogenic bacterial growth (Sugita et al., 1996) as well as ability to ferment peptides, a important characteristic for carnivorous fish (Romero et al., 2014).

Linear discriminant analysis (LDA) and effect size (LefSE) results show the bacterial groups that drove the main differences between treatments. Within the class Bacilli, reads assigned as Lactobacillaceae family and Lactobacillales order were significantly different in fish intestinal mucosa fed with probiotic *Lactococcus lactis* subsp. *lactis* (LacM) in the present study. Ringø and Gatesoupe (1998) described

Bacilli, especially lactobacilli, as members of fish gut microbiota. They are able to ferment carbohydrates, however, are not able to produce extracellular degradative enzymes. *Lactobacillus* sp. are known lactic acid bacteria used as probiotic in different fish species such as rainbow trout (*O. mykiss*) (Balcázar et al., 2007b; Nikoskelainen et al., 2003), brown trout (*Salmo trutta*) (Balcazar et al., 2007) and Nile tilapia (*O. niloticus*). On the other hand, the genus *Pseudomonas* was significantly different in this same treatment (LacM). Although some authors report the presence of *Pseudomonas* in gut of some fish such as zebrafish (*Danio rerio*) (Roeselers et al., 2011) and rainbow trout (*O. mykiss*) (Etyemez and Balcázar, 2015), many species within *Pseudomonas* genus were related with fish disease (Colquhoun et al., 1998; Jang et al., 2014; Xu et al., 2015). Thus, the presence of *Pseudomonas* in the tract of fish under this treatment is not a pleasant result because they are known as opportunistic bacteria and can take advantage of an unbalance of microbiota causing possible intestinal infections.

The significant difference of *Enterococcus* sp. in intestinal mucosa of fish fed with probiotic *Enterococcus faecium* (EntM) (after LEfSE analysis) could be an indication that this bacterium was able to colonise the fish mucosal tissue. However, some authors characterise probiotic colonization if the probiont is able to maintain a population for significant periods of time after the cessation of probiotic feeding (Merrifield and Carnevali, 2014). Additionally, RT-qPCR results attested that intestinal mucosa of fish fed with probiotic *Enterococcus faecium* (EntM) had more concentration of DNA of *Enterococcus* sp. when compared to fish that did not receive control diet. The concentration of this strain actually increased 100x from the original probiotic concentration ($10^7$ CFU.ml$^{-1}$) available in the fish feed. Indeed the probiotic *Enterococcus faecium* was responsible for the main differences in microbiota under this specific treatment after populating the mucosal intestine tissue in *A. gigas*. however, the term "populate" would be more appropriate for this result instead of "colonised" (Merrifield and Carnevali, 2014).

Alpha diversity parameters analysis calculates species richness within the community, which includes Chao1 index, number of observed species and phylogenetic diversity (PD). Within Alpha diversity, PD of mucosal samples was different from digesta samples in the present study. Mucosal samples appear to be richer than digesta samples in all treatments analysed. Falcinelli et al. (2015) found phylogenetic diversity in zebrafish gut treated with probiotic lower when compared

with control fish. However, the difference observed in the present study was driven by difference of tissue (mucosa x digesta) and not due to probiotic feeding.

Beta diversity parameters (PCoA plots) illustrate the differences between samples and support the idea described previously. Mucosa samples are clustered together as well as digesta samples for Unweighted Unifrac. These results are also supported by few differences in PERMANOVA (Table 7). Due to low diversity across the treatments, the core microbiota was high among the treatments for mucosa (52.8%) and digesta (60%).

*Physiological variables after probiotic feeding*

In contrast to the modulation of microbiota in which the main differences where observed under Enterococcus-fed treatment, the probiotic *Lactococcus lactis* subsp. *lactis* however drove the main physiological changes observed in *A. gigas*.

For instance, specific growth rate (SGR) and percentage of increase (I%) in weight were higher in *A. gigas* fed with the probiotic *L. lactis* subsp. *lactis*. This suggest that the probiotic administration might be inducing the activity of digestive enzymes in the digestive tract, eventually improving growth parameters (Dimitroglou et al., 2011). However, this conclusion could be speculative since there could be other mechanisms that influence growth performance resulting from another microbe changing activity or abundance. Other authors demonstrated similar results in fish fed with this strain. Nguyen et al. (2017) reported probiotic strain *L. lactis* WFLU12 significantly improved olive flounder (*Paralichthys olivaceus*) growth, feed conversion, and specific growth rate after 30 days and 60 days of feeding. Likewise, the dietary supplementation of a probiotic mix containing *Lactococcus lactis* during 56 days enhanced growth performance and feed utilization of red sea bream (*Pagrus major*) (Dawood et al., 2016). One of the main adavantages of probiotic administration in fish is the improvement of growth performance. In a production point of view, this is advantageous because the FCR and other growth parameters such as SGR and percentage of weight increase could be improved with probiotics administration. However, growth parameters may not be used alone as an indicator of health improvement. Thus, other physiological variables such as gut morphology and hsemato-immunological parameters should be analysed together.

The study of blood components is helpful to understand comparative physiology between healty and diseased fish, fish under different feeding, fish held in

different captive conditions, fish exposed to different treatments or fish living in different habitats. The monocytes concentration in blood of fish from the treatment fed with *L. lactis* subsp. *lactis* in the present study was significant higher when compared with other two treatments. Probiotics have previously been reported to influence the abundance or activity of immune cells such as monocytes (mononuclear phagocytic cells) (Nayak, 2010). Similar results were found in rohu (*Labeo rohita*) after probiotic *Bacillus subtilis* diet administration (Kumar et al., 2008). Cellular stimulation and monocytes increased concentration was also observed in rainbow trout (*O. mykiss*) after receiving different probiotic strains in diet (Irianto and Austin, 2002b). Monocytes are key components of the innate immune system in fish and are responsible for pathogen phagocytosis as well as differentiate into macrophages in order to migrate to an inflammatory site as a defense response (Bailone et al., 2010; Standen et al., 2013b). Thus, the probiotic could be contributing with fish immune response helping the fish to be prepared to face a possible infection eventually.

The current findings and support the idea that the probiotic *L. lactis* subsp. *lactis* used in the present study influenced some physiological variables such as growth and haematological parameters.

## 5.6 Conclusion

The probiotic strain *Enterococcus faecium* interacted with microbial gut community in pirarucu and was able to keep populate the mucosal intestinal tissue under the conditions tested in the present chapter. The effects of *Lactococcus lactis* subsp. *lactis* on the gut microbiota of pirarucu was less evident. However, this did not prevent it from having impacts on *A. gigas* t cellular immune parameters and growth parameters. These results suggest that both strains tested in the present study improved the health status and production in *A. gigas*, and could be used in pirarucu production in the future.

# Chapter 6:
# General Discussion

# Chapter 6: General Discussion

This thesis describes for the first time a comprehensive map of intestinal microbiota in pirarucu using HTS technology as well as evidence of the potential of authochthonous probiotics in both *in vitro* and *in vivo* experiments. In general, both potential probiotic isolates *Enterococcus faecium* and *Lactococcus lactis* subsp. *lactis* have were demonstrated to be able to modulate intestinal microbiota in pirarucu (*Arapaima gigas*). Separately, *E. faecium* could adhere to intestinal mucosa and decrease pathogenic bacteria levels in the intestinal tract under the experimental conditions described in the present study. In addition, *L. lactis* subsp. *lactis* was able to modulate immunological parameters and induce improved growth performance during the experiments performed.

## 6.1 Modulation of the intestinal microbiota

During recent years, the application of HTS in aquaculture research became a reality. The "omics" tools, including metagenomics, proteomics, transcriptomics, have been employed to characterize and understand the bacterial microbiomes of fish tract and their interaction with fish immunological tissues (Salinas and Magadán, 2017). The composition of bacterial community in different fish species under different conditions, such as: diet formulation, environmental factors (wild x captive) and rearing temperature were recently published (Gajardo et al., 2016; Ghanbari et al., 2015; Parma et al., 2016; Tarnecki et al., 2017). However, to the author's knowledge, few studies have analysed the modulation of microbiota after probiotic feeding or feed additives administration are available in the literature.

**General findings**

Pirarucu intestinal microbiota seems to be similar to other fish from a general perspective. Fusobacteria, Proteobacteria and Firmicutes were the most abundant phyla in pirarucu distal intestine and were also reported as being abundant in different fish species (Llewellyn et al., 2014; Tarnecki et al., 2017). Interestingly, when analysing the abundance of microbiota at phylum level of pirarucu under *normal* rearing condition (non-supplemented with probiotic in Chapter 3a) the phylum Proteobacteria was less abundant in juvenile fish and increased with maturity, being more abundant in adult fish. This same phylum showed low abundance in both

experiments with probiotic feeding (Chapters 4 and 5), even in controls groups (non-probiotic fed fish). In fact, the intestinal gut microbiota is very dynamic and can vary according many different conditions such as host factors (age, sex, species), different diet formulations (lipid, protein and carbohydrates content) and feed additives (probiotics, prebiotics and immunostimulants) (Dimitroglou et al., 2011).

Samples to perform bacterial community profiling under *normal* conditions of pirarucu were taken in the same fish farm located in Rondonia state (Brazil), however, during different season and years. In addition, the fish from Chapter 3a were sampled from ponds; normal rearing conditions for this fish farm, while the fish from both probiotic experiments were maintained in 1000 l tanks. Some authors have reported a high abundance of Proteobacteria in the gut of wild fish species with different feeding habits (Givens et al., 2015; Li et al., 2014). Within this phylum, the γ-Proteobacteria representatives such as *Aeromonas* spp., *Escherichia coli*, *Photobacterium* spp., *Pseudomonas* spp., and *Vibrio* spp. are normally found in water or soil (Gauthier, 2015). This may partly explain why this phylum was not abundant during the probiotic experiments, because they were performed using tanks as experimental replicates and not performed in earth ponds.

In the present study, it is important to highlight the genus *Cetobacterium*. This genus was highly abundant in all pirarucu across different treatments and rearing conditions. *Cetobacterium* sp. have characteristics already discussed in the previous chapters such as ability to inhibit the growth of potential pathogenic bacteria, peptide fermentation and production of vitamin B12 (Romero et al., 2014; Sugita et al., 1996). Although its applications in fish microbiota interaction are still unknown, the characteristics cited above reveals the importance of this genus for pirarucu nutrition. For instance, *Cetobacterium somerae* isolated from human faeces are bile resistant and produce acetic acid as product of metabolism of peptides and carbohydrates (Finegold et al., 2003). This strain is also known to be an aero-tolerant anaerobe and was previously identified as being abundant in biofilms of tilapia (*Oreochromis niloticus*) rearing tanks (Burgos et al., 2018). Furthermore, it was reported to be vancomycin resistant (Tsuchiya et al., 2008), however, to the authors knowledge, any further evidence of resistance was described in *Cetobacterium* strains directly isolated from fish.

Amongst the factors that could influence modulation of microbiota cited previously, the host factor such as age was found to drive differences in pirarucu

intestine in the present study. There was a clear difference between juvenile and adult fish microbiota in chapter 3a (PcoA plots). The shift of microbiota according to the fish age was also reported in pinfish (*Lagodon rhomboids*) (Givens et al., 2015) and zebrafish (*Danio rerio*) (Rurangwa et al., 2015; Stephens et al., 2016; Wong et al., 2015). This modification of microbiota is possibly related with the shift of diet during the ontogenetic development of fish and could also possible be related with the change of environment in (rearing conditions) in captive fish (Stephens et al., 2016). Thus, this change of microbiota according to the fish development stage is likely to be normal and should be taken into account when HTS analysis is performed.

**Probiotic induced modulation of the intestinal microbiota**

The two strains *Enterococcus faecium* and *Lactococcus lactis* subsp. *lactis* isolated during the present study showed ability to modulate pirarucu intestinal microbiota under both experimental conditions (Chapter 4 and 5), however, the dose of administration drove some differences between results of both experimental chapters.

For instance, results in chaper 4 describe the decrease of abundance of the class Clostridia in the gut of fish that received probitics at $10^8$ CFU.g$^{-1}$ during 21 days as well as the presence of sequences assigned as genus *Enterococcus* and *Lactococcus* in gut of fish suggesting that the probiotic was able to compete for adhesion site in fish gut. However, when pirarucu were fed with the same autochthonous strains at $10^7$ CFU.g$^{-1}$ during 42 days, the modulation of microbiota was less evident. It is clear that the difference of concentration used in both chapters is at least partially responsible for the differeing extent of the influenceon microbiota modulation by the probiotic strains. Nevertheless, the strain *E. faecium* was not afftected by the concentration of administration and was still detected in *A. gigas* gut when administrated at $10^7$ CFU.g$^{-1}$ of diet in chapter 5.

The genus *Enterococcus* was also detected in high abundance in tilapia (*O. niloticus*) fed with a commercial multi-strain probiotic, containing *E. faecium* (AquaStar® Growout) when compared with fish not fed with probiotic in diet (Standen et al., 2015) corroborating with the findings of pirarucu. The reduction of viable counts of harmful bacteria such as *Staphylococcus aureus*, *Escherichia coli* and Enterobacteriaceae with complete elimination of *Clostridium* spp. after 2 weeks of

feeding was also observed in sheat fish (*Silurus glanis*) fed with *E. faecium* at the concentration of $2x10^8$ CFU.$g^{-1}$ of diet (Bogut et al., 2000).

On the other hand, the class Bacilli was significantly different in fish intestinal mucosa fed with the strain *L. lactis* subsp. *lactis* even at lower concentration. The increase of Lactobacillaceae family and decrease of Actinobacteria in fish gut fed with *L. lactis* when compared to control fish suggests that this strain was also able to modulated the some representative of bacterial community under the experimental conditions.

Although interestingly and desirable, few authors described the modulation of microbiota or microbial community after probiotic feeding using HTS tools. The exceptions are the limited studies available for zebrafish (*Danio rerio*) (Falcinelli et al., 2017, 2016), tilapia (*O. niloticus*) (Adeoye et al., 2016; Standen et al., 2015) and rainbow trout (*O. mykiss*) (Gonçalves et al., 2017). More research is therefore required on the topic of modulation of microbiota linked with probiotic feeding in order to improve knowledge about the interaction between gut bacterial community and physiological status in fish.

## 6.2 Probiotic colonization

According Conway (1996), microbial colonization occurs when a microorganism is able to keep alive for a certain time in the intestinal tract, however, in aquatic animals it is more dynamic than terrestrial animals. Balcazar et al. (2006) has described probiotic colonisation as the "attraction of bacteria to the mucosal surface, followed by association within the mucous gel or attachment to epithelial cells."

As described previously, the autochthonous strains *Enterococcus faecium* and *Lactococcus lactis* subsp. *lactis* were detected in mucosa of fish fed with probiotics $10^8$ CFU.$g^{-1}$ during 21 days (Chapter 4). On the other hand, they have shown different behaviour when administered at the concentration of $10^7$ CFU.$g^{-1}$ during 42 days (Chapter 5). The strain *E. faecium* was still present in mucosal tissue; however, the strain *L. lactis* subsp. *lactis* was not detected in mucosal tissue after HTS analysis. It seems this strain of *L. lactis* was not able to adhere on the mucosa after the dosage of $10^7$ CFU.$g^{-1}$ and time of 42 days during the experiment. However, further experiments are required to ascertain if this was driven mostly by time or dose.

The adhesion of mucosal tissues might have a protective mechanism against pathogens by competition of binding sites and source of nutrients as well as gastrointestinal immune modulation (Balcazar et al., 2006). However, this adhesion seems to be related with the dose of administration of probiotics. Thus, the administration dosage is one of the factors that can vary according to probiont species. Factors such as probiotic concentration (dose) and low administration time play a role in the efficacy of probiotic colonization, adherence and also physiological and immunological modulation in fish (Fečkaninová et al., 2017). Another viariant that influence on the probiotic is the temporal effect or the time that a probiotic is administrated (Lauzon et al., 2010).

For instance, the administration of *B. subtilis* at $10^8$ and $10^7$ CFU.g$^{-1}$ of diet showed higher modulation of immunological parameters and growth when compared with *L. plantarum* at same concentrations in Japanese eel (*Anguilla japonica*) (Lee et al., 2017). In addition, different fish species could require different dosages of probiotic. For example, *L. lactis* subsp. *lactis* at concentration of $10^6$ CFU.g$^{-1}$ of diet was used as probiotic in brown trout (*Salmo trutta*) during 15 days. Differently from the present study, in brown trout, the feeding time of two weeks of *L. lactis* at this concentration was sufficient to modulate immunological parameters and persist viable up to two weeks after cessation of probiotic. Regarding to the present study, the adherence ability of both probiotic isolates *Enterococcus faecium* and *Lactococcus lactis* subsp. *lactis* seems to be more effective at the concentration of $10^8$ CFU.g$^{-1}$.

Merrifield and Carnevali (2014) discussed that the recovery of the probiont after a dietary supplementation is not evidence of colonization *per se*. Therefore, the word *colonization* should be associated with the capacity of the probiont to multiply inside the intestinal tract and present higher levels than the administrated concentration in diet. Only, few studies have reported the levels of probiotic bacteria in fish gut higher than the feeding level (Balcázar et al., 2007a, 2007b; Bucio Galindo et al., 2009). Thus, the word *adhere* seems to be used when the works only detect the levels of probiotic after a feeding trial and the term *recovery* should be used when the levels of bacteria are detected after ceasetion of probiotic intake.

Previous publications have reported recovery of some probiotic bacteria in fish gut. Probiotics such as *Carnobacterium divergens* and *C. maltaromaticum* were still identified for three weeks in rainbow trout (*Oncorhynchus mykiss*) intestine after probiotic feeding ceased (Kim and Austin, 2006). However, *Lactobacillus plantarum*

was not detected in tilapia (*Oreochromis niloticus*) intestine three days after cessation of probiotic intake (Bucio Galindo et al., 2009). Although these two studies used different methodology to detect the presence of probiotic after a cessation period, they show that the recovery of the probiont is different than the colonization of the gut. Therefore, the presence of the target probiotic in the mucosal tissue should be further studied to ascertain colonization (Romero et al., 2014).

The results of this thesis suggest that there is a difference in probiotic abundance and recovery when isolated strains of *Enterococcus* sp. and *Lactococcus* sp. are administered using two different concentrations and timescales. Both strains were able to modulate microbiota and decrease levels of pathogenic bacteria in intestinal tract. In this context, further understanding of the interactions between these two authochtonous strains is needed to assess the effects in the fish gut microbiota and colonization. Testing a probiotic mixture with both strains would allow determining potential synergism or antagonism between them, which should be considered in fish diet formulations.

## 6.3 Physiological parameters

In the present thesis, the different probiotic candidates were able to improve distinctive parameters in pirarucu. For instance, eosinophil concentration in blood was higher in fish fed with *Enterococcus faecium* for 21 days (Chapter 4), while monocytes concentration was higher in fish fed with *Lactococcus lactis* subsp. *lactis* for 42 days (Chapter 5). However, the strain *L lactis* subsp. *lactis* seems to have a role in immunological parameters such as higher serum antimicrobial activity (Chapter 4) as well as growth parameters such as higher specific growth rate (SGR) and percentage of increase (%I) (Chapter 5).

Previous authors have reported similar results. The dietary administration of *Lactococcus lactis* itself seems to driven more the physiological factors such as growth in sea bream (*Pagrus major*), but the combination of *L. lactis* and *Lactobacillus rhamnosus* were more effective in immunological modulation of sea bream after 56 days of probiotic feeding (Dawood et al., 2016). Lin et al. (2017) also reported different results linked to different bacteria. When administered separately, the strains *Bacillus subtilis* E20 and *Lactobacillus pentosus* BD6 in diet for 30 days promoted the growth of the Asian seabass (*Lates calcarifer*). On the other hand, the

strains *Saccharomyces cerevisiae* P13 and *Lactobacillus fermentum* LW2 improved the health status of *L. calcarifer*. A synergistic effect of combined probiotics containing the four strains cited previously improved both the growth performance and health of Asian seabass in this study.

Interestingly, both authors cited in the previously paragraph described synergistic effect of combined probiotics in both fish species. Indeed there is a combined effect in fish growth and health improvement when fish were fed with the probiotic strains named in these two studies (Dawood et al., 2016; Lin et al., 2017). However, further *in vitro* analysis should be executed to assure that both strains isolated from pirarucu (*A. gigas*) do not have antagonistic effect between each other. Also, further *in vivo* experiments should be performed to detect if there is synergistic effect in growth and immunological status when *Lactococcus lactis* subsp. *lactis* and *Enterococcus faecium* are used combined.

## 6.5 Future research efforts

According FAO (2015), the production of *Arapaima gigas* in Brazil started being significant in the last 15 years. Thus, there is still a lack of knowledge on the best methods for pirarucu industrial production including specific nutritional requirements for diets, assessment of feed additives, and diagnosis of pathogens. However, the prelimirary output of this thesis helps in the development of techonological pack Brazilian native species.

For example, the isolation of probiotic in the present thesis was performed following standard protocols previously published by other authors. However, the use of only one culture medium, in this case, the MRS, focused on the isolation LAB. Therefore, the isolation of bacteria such as *Bacillus* or *Cetobacterium* was not possible. Thus, this thesis represents one group of the possible probiotic bacteria that could be found in *A. gigas* intestine. Additionally, the high abundance of the genus *Cetobacterium* revealed by HTS analysis highlights the importance of this strain for freshwater fish species and emphasizes a need for future research on this specific strain. However, this strain is not likely to be considered a viable probiotic because it is too difficult to culture and thus not economically friendly.

Another important topic is the probiotic concentration. In the present study probiotics at $10^7$ CFU.$g^{-1}$ was found to be insufficient to achieve probiotic populations

on the intestinal mucosa and decrease pathogenic bacteria. Thus, the optimal administration dosage as well as feeding period time is still unclear and should be investigated using a dose response experimental design. It may also be possible that multi-strain application of both strains simultaneously could yield beneficial results and should be explored, along with potential combinations with prebiotics to assess the viability of symbiotic applications.

During laboratory work of the present thesis, it was possible to extract RNA from pirarucu intestine and amplify reference genes such as: elongator factor (EF1- α) and beta actin (β-actin). However, the quantitation of target genes for anti-inflamatory and pro-inflamatory responses was not successful. Indeed, there is still a lack of knowledge on specific genes for in *A. gigas*. For instance, the genome for this specie was not sequenced yet. Consequently, there is no information on genes regulation after probiotic feeding described in the literature for this fish. Thus, the primers used for this analysis were designed based on genes available in NCBI from phylogenetic related fish such as catfish (*Ictalurus puntactus*) and sturgeon (*Acipenser transmontanus*). Finally, the expression of genes in *A. gigas* intestine was not possible in the current study and future research on expressed genes in pirarucu is necessary to the interaction between protiotics and genes dynamic in pirarucu's gut.

Despite these knowledge gaps, the data presented in this thesis are novel and informative and thus make a positive contribution to the field of probiotic research in fish. The isolation of two bacterial strains with no antibiotic resistant genes for at least three of the antibiotic currently used in aquaculture is a good starting point for helping to drive the sustainable production of pirarucu in Brazil. The results of this thesis suggest that the strains promoted growth performance, improved immunological parameters and modulate intestinal microbiota in fish gut. These physiological characteristics together with the decrease use of antibiotics in pirarucu farms could improve marketing and add value on the fish as a final sustainable aquaculture product in the future.

# References

## References:


Abid, A., Davies, S.J., Waines, P., Emery, M., Castex, M., Gioacchini, G., Carnevali, O., Bickerdike, R., Romero, J., Merrifield, D.L., 2013. Dietary synbiotic application modulates Atlantic salmon (*Salmo salar*) intestinal microbial communities and intestinal immunity. Fish Shellfish Immunol. 35, 1948–1956. doi:http://dx.doi.org/10.1016/j.fsi.2013.09.039

Adeoye, A.A., Yomla, R., Jaramillo-Torres, A., Rodiles, A., Merrifield, D.L., Davies, S.J., 2016. Combined effects of exogenous enzymes and probiotic on Nile tilapia (*Oreochromis niloticus*) growth, intestinal morphology and microbiome. Aquaculture 463, 61–70. doi:http://dx.doi.org/10.1016/j.aquaculture.2016.05.028

Al-Dohail, M.A., Hashim, R., Aliyu-Paiko, M., 2009. Effects of the probiotic, *Lactobacillus acidophilus*, on the growth performance, haematology parameters and immunoglobulin concentration in African Catfish (*Clarias gariepinus,* Burchell 1822) fingerling. Aquac. Res. 40, 1642–1652. doi:10.1111/j.1365-2109.2009.02265.x

Al-Hisnawi, A., Ringø, E., Davies, S.J., Waines, P., Bradley, G., Merrifield, D.L., 2014. First report on the autochthonous gut microbiota of brown trout (*Salmo trutta* Linnaeus). Aquac. Res. 2962–2971. doi:10.1111/are.12451

Aly, S.M., Abdel-Galil Ahmed, Y., Abdel-Aziz Ghareeb, A., Mohamed, M.F., 2008. Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of Tilapia nilotica (*Oreochromis niloticus*) to challenge infections. Fish Shellfish Immunol. 25, 128–136. doi:http://dx.doi.org/10.1016/j.fsi.2008.03.013

Ammor, M.S., Belén Flórez, A., Mayo, B., 2007. Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. Food Microbiol. 24, 559–570. doi:http://dx.doi.org/10.1016/j.fm.2006.11.001

AOAC. Official Methods of Analysis, 18o ed. Association of Official Analytical Chemists, Gaithersburg, 2005.

Araújo, C., Muñoz-Atienza, E., Poeta, P., Igrejas, G., PE, H., Herranz, C., 2016. Characterization of P*ediococcus acidilactici* strains isolated from rainbow trout (*Oncorhynchus mykiss*) feed and larvae: safety, DNA fingerprinting, and bacteriocinogenicity . Dis. Aquat. Organ. 119, 129–143.

Askarian, F., Zhou, Z., Olsen, R.E., Sperstad, S., Ringø, E., 2012. Culturable autochthonous gut bacteria in Atlantic salmon (*Salmo salar* L.) fed diets with or without chitin. Characterization by 16S rRNA gene sequencing, ability to produce enzymes and in vitro growth inhibition of four fish pathogens. Aquaculture 326, 1–8. doi:http://dx.doi.org/10.1016/j.aquaculture.2011.10.016

Attia A  Galil YA, Fathi M, M.S., 2012. Effect of Injection Vaccination against *Pseudomonas fluorescens* on Specific and Non-Specific Immune Response of Nile Tilapia (*Oreochromis niloticus*) Using Different Prepared Antigens. Open Access Sci. Reports 1, 1–7. doi:10.4172/scientificreports.552

Austin, B., Austin, D.A., 2007. Bacterial fish pathogens : disease of farmed and wild fish, 4rd (rev.). ed. London : Springer.

Avella, M.A., Olivotto, I., Silvi, S., Ribecco, C., Cresci, A., Palermo, F., Polzonetti, A., Carnevali, O., 2011. Use of *Enterococcus faecium* to improve common sole

(*Solea solea*) larviculture. Aquaculture 315, 384–393. doi:http://dx.doi.org/10.1016/j.aquaculture.2011.02.046

Azevedo, R.V. de, Fosse Filho, J.C., Pereira, S.L., Cardoso, L.D., Vidal Júnior, M.V., Andrade, D.R. de, 2016. Suplementação com prebiótico, probiótico e simbiótico para juvenis de tambaqui a duas densidades de estocagem . Pesqui. Agropecuária Bras. 51, 9-16 DOI: 10.1590/S0100-204X2016000100002

Azimirad, M., Meshkini, S., Ahmadifard, N., Hoseinifar, S.H., 2016. The effects of feeding with synbiotic (*Pediococcus acidilactici* and fructooligosaccharide) enriched adult Artemia on skin mucus immune responses, stress resistance, intestinal microbiota and performance of angelfish (*Pterophyllum scalare*). Fish Shellfish Immunol. 54, 516–522. doi:https://doi.org/10.1016/j.fsi.2016.05.001

Bailone, R.L., Martins, M.L., Mouriño, J.L.P., Vieira, F.N., Pedrotti, F.S., Nunes, G.C., Silva, B.C., 2010. Hematology and agglutination titer after polyvalent immunization and subsequent challenge with *Aeromonas hydrophila* in Nile tilapia (*Oreochromis niloticus*). Arch. Med. Vet. 42, 221–227. doi:10.4067/S0301-732X2010000300015

Balcázar, J.L., Blas, I. de, Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., Múzquiz, J.L., 2006. The role of probiotics in aquaculture. Vet. Microbiol. 114, 173–186. doi:http://dx.doi.org/10.1016/j.vetmic.2006.01.009

Balcazar, J.L., de Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., Muzquiz, J.L., 2006. The role of probiotics in aquaculture. Vet. Microbiol. 114, 173–186. doi: 10.1016/j.vetmic.2006.01.009

Balcazar, J.L., De Blas, I., Ruiz-Zarzuela, I., Vendrell, D., Calvo, A.C., Marquez, I., Girones, O., Muzquiz, J.L., 2007. Changes in intestinal microbiota and humoral immune response following probiotic administration in brown trout (*Salmo trutta*). Br J Nutr 97, 522–527. doi:10.1017/S0007114507432986

Balcázar, J.L., de Blas, I., Ruiz-Zarzuela, I., Vendrell, D., Calvo, A.C., Márquez, I., Gironés, O., Muzquiz, J.L., 2007a. Changes in intestinal microbiota and humoral immune response following probiotic administration in brown trout (*Salmo trutta*). Br. J. Nutr. 97, 522. doi:10.1017/S0007114507432986

Balcázar, J.L., De Blas, I., Ruiz-Zarzuela, I., Vendrell, D., Gironés, O., Muzquiz, J.L., 2007b. Enhancement of the immune response and protection induced by probiotic lactic acid bacteria against furunculosis in rainbow trout (*Oncorhynchus mykiss*). FEMS Immunol. Med. Microbiol. 51, 185–193. doi:10.1111/j.1574-695X.2007.00294.x

Baldisserotto, B., Copatti, C.E., Gomes, L.C., Chagas, E.C., Brinn, R.P., Roubach, R., 2008. Net ion fluxes in the facultative air-breather *Hoplosternum littorale* (tamoata) and the obligate air-breather *Arapaima gigas* (pirarucu) exposed to different Amazonian waters. Fish Physiol. Biochem. 34, 405–412. doi:10.1007/s10695-008-9200-y

Banerjee, G., Ray, A.K., 2017. The advancement of probiotics research and its application in fish farming industries. Res. Vet. Sci. 115, 66–77. doi:https://doi.org/10.1016/j.rvsc.2017.01.016

Barbosa, M.C., Jatobá, A., Vieira, F. do N., Silva, B.C., Mourino, J.L.P., Andreatta, E.R., Seiffert, W.Q., Cerqueira, V.R., 2011. Cultivation of juvenile fat snook (*Centropomus parallelus* Poey, 1860) fed probiotic in laboratory conditions.

Brazilian Arch. Biol. Technol. 54, 795–801.

Barcellos, L.J.G., Kreutz, L.C., Quevedo, R.M., Fioreze, I., Cericato, L., Soso, A.B., Fagundes, M., Conrad, J., Baldissera, R.K., Bruschi, A., Ritter, F., 2004. Nursery rearing of jundiá, *Rhamdia quelen* (Quoy & Gaimard) in cages: cage type, stocking density and stress response to confinement. Aquaculture 232, 383–394. doi:http://dx.doi.org/10.1016/S0044-8486(03)00545-3

Bastardo, A., Ravelo, C., Castro, N., Calheiros, J., Romalde, J.L., 2012. Effectiveness of bivalent vaccines against *Aeromonas hydrophila* and *Lactococcus garvieae* infections in rainbow trout *Oncorhynchus mykiss* (Walbaum). Fish Shellfish Immunol. doi:S1050-4648(12)00039-3 [pii]10.1016/j.fsi.2012.01.028

Bezerra, R.F., Soares, M. do C.F., Carvalho, E.M., Coelho, L.C.B.B., 2013. Pirarucu, *Arapaima gigas*, the Amazonian Giant Fish is Briefly Reviewed, Fish, Fish. ed. Nova Science Publishers, Inc., New York.

Bogut, I., Milaković, Z., Brkić, S., Novoselić, D., Bukvić, Ž., 2000. Effects of *Enterococcus faecium* on the growth rate and content of intestinal microflora in sheat fish (*Silurus glanis*). Vet. Med. (Praha). 45, 107–109.

Bogut, I., Milakovic, Z., Bukvic, Z., Brkic, S., Zimmer, R., 1998. Influence of probiotic (*Streptococcus faecium* M74) on growth and content of intestinal microflora in carp (*Cyprinus carpio*). Czech J. Anim. Sci. (Czech Republic).

Brauner, C.J., Matey, V., Wilson, J.M., Bernier, N.J., Val, A.L., 2004. Transition in organ function during the evolution of air-breathing; insights from *Arapaima gigas*, an obligate air-breathing teleost from the Amazon. J. Exp. Biol. 207, 1433–1438. doi:Doi 10.1242/Jeb.00887

Broughton, R.E., Betancur-R., R., Li, C., Arratia, G., Ortí, G., 2013. Multi-locus phylogenetic analysis reveals the pattern and tempo of bony fish evolution. PLoS Curr. 5. doi:10.1371/currents.tol.2ca8041495ffafd0c92756e75247483e

Bucio Galindo, A., Hartemink, R., Schrama, J.W., Verreth, J., Bucio G, L., Zwietering, M.H., 2009. Kinetics of *Lactobacillus plantarum* 44a in the faeces of tilapia (*Oreochromis niloticus*) after its intake in feed. J. Appl. Microbiol. 107, 1967–1975. doi:10.1111/j.1365-2672.2009.04382.x

Burgos, M.J.G., Romero, J.L., Pérez Pulido, R., Cobo Molinos, A., Gálvez, A., Lucas, R., 2018. Analysis of potential risks from the bacterial communities associated with air-contact surfaces from tilapia (*Oreochromis niloticus*) fish farming. Environ. Res. 160, 385–390. doi:https://doi.org/10.1016/j.envres.2017.10.021

Burton, A.M., Calderero, E.M., Moran, R.E.B., Sánchez, R.L.A., Villamar, U.T.A., Torres, N.G.O., 2016. A simple and low-cost recirculating aquaculture system for the production of arapaima juveniles. Rev. Int. Investig. y Docencia 1, 49.

Caporaso, J.G., Bittinger, K., Bushman, F.D., DeSantis, T.Z., Andersen, G.L., Knight, R., 2010a. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26, 266–267. doi:10.1093/bioinformatics/btp636

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010b. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7,

335–336. doi:10.1038/nmeth.f.303

Carnevali, O., Avella, M.A., Gioacchini, G., 2013. Effects of probiotic administration on zebrafish development and reproduction. Gen. Comp. Endocrinol. 188, 297–302. doi:https://doi.org/10.1016/j.ygcen.2013.02.022

Carnevali, O., Maradonna, F., Gioacchini, G., 2017. Integrated control of fish metabolism, wellbeing and reproduction: The role of probiotic. Aquaculture 472, 144–155. doi:https://doi.org/10.1016/j.aquaculture.2016.03.037

Castro, R., Tafalla, C., 2015. 2 - Overview of fish immunity, in: Peatman, B.H.B. (Ed.), Mucosal Health in Aquaculture. Academic Press, San Diego, pp. 3–54. doi:http://dx.doi.org/10.1016/B978-0-12-417186-2.00002-9

Cavero, B.A.S., Pereira-Filho, M., Bordinhon, A.M., Fonseca, F.A.L. da, Ituassú, D.R., Roubach, R., Ono, E.A., 2004. Tolerância de juvenis de pirarucu ao aumento da concentração de amônia em ambiente confinado. Pesqui. Agropecu. Bras. 39, 513–516.

Chang, C.I., Liu, W.Y., 2002. An evaluation of two probiotic bacterial strains, Enterococcus faecium SF68 and Bacillus toyoi, for reducing edwardsiellosis in cultured European eel, Anguilla anguilla L. J. Fish Dis. 25, 311–315. doi:DOI 10.1046/j.1365-2761.2002.00365.x

Chi, C., Jiang, B., Yu, X.-B., Liu, T.-Q., Xia, L., Wang, G.-X., 2014. Effects of three strains of intestinal autochthonous bacteria and their extracellular products on the immune response and disease resistance of common carp, *Cyprinus carpio*. Fish Shellfish Immunol. 36, 9–18. doi:http://dx.doi.org/10.1016/j.fsi.2013.10.003

Colquhoun, D.J., Skjerve, E., Poppe, T.T., 1998. *Pseudomonas fluorescens*, infectious pancreatic necrosis virus and environmental stress as potential factors in the development of vaccine related adhesions in Atlantic salmon, *Salmo salar* L. J. Fish Dis. 21, 355–363.

Coppola, M.D., Conceicao, F.R., Gil-Turnes, C., 2005. Effect of *Saccharomyces boulardii* and *Bacillus cereus* var. *toyoi* on the humoral and cellular response of mice to vaccines. Food Agric. Immunol. 16, 213–219. doi:Doi 10.1080/09540100500244138

Currell, G., 2015. Scientific data analysis. Oxford University Press (UK).

Cyrino, J.E.P., Bicudo, Á.J. de A., Sado, R.Y., Borghesi, R., Dairik, J.K., 2010. A piscicultura e o ambiente: o uso de alimentos ambientalmente corretos em piscicultura . Rev. Bras. Zootec. 39, 68-87. http://dx.doi.org/10.1590/S1516-35982010001300009.

da Silva, B.C., Mourino, J.L.P., Vieira, F.N., Jatoba, A., Seiffert, W.Q., Martins, M.L., 2012. Haemorrhagic septicaemia in the hybrid surubim (*Pseudoplatystoma corruscans* x *Pseudoplatystoma fasciatum*) caused by *Aeromonas hydrophila*. Aquac. Res. 43, 908–916. doi:DOI 10.1111/j.1365-2109.2011.02905.x

Dalmo, R.A., Ingebrigtsen, K., Bogwald, J., 1997. Non-specific defence mechanisms in fish, with particular reference to the reticuloendothelial system (RES). J. Fish Dis. 20, 241–273. doi: 10.1046/j.1365-2761.1997.00302.x

Das, A., Nakhro, K., Chowdhury, S., Kamilya, D., 2013. Effects of potential probiotic Bacillus amyloliquifaciens FPTB16 on systemic and cutaneous mucosal immune responses and disease resistance of catla (*Catla catla*). Fish Shellfish Immunol.

35, 1547–1553. doi:http://dx.doi.org/10.1016/j.fsi.2013.08.022

Dawood, M.A.O., Koshio, S., Ishikawa, M., Yokoyama, S., El Basuini, M.F., Hossain, M.S., Nhu, T.H., Dossou, S., Moss, A.S., 2016. Effects of dietary supplementation of *Lactobacillus rhamnosus* or/and *Lactococcus lactis* on the growth, gut microbiota and immune responses of red sea bream, *Pagrus major*. Fish Shellfish Immunol. 49, 275–285. doi:https://doi.org/10.1016/j.fsi.2015.12.047

de Andrade, J.I.A., Ono, E.A., de Menezes, G.C., Brasil, E.M., Roubach, R., Urbinati, E.C., Tavares-Dias, M., Marcon, J.L., Affonso, E.G., 2007. Influence of diets supplemented with vitamins C and E on pirarucu (*Arapaima gigas*) blood parameters. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 146, 576–580. doi:http://dx.doi.org/10.1016/j.cbpa.2006.03.017

de Oliveira, E.G., Pinheiro, A.B., de Oliveira, V.Q., da Silva Júnior, A.R.M., de Moraes, M.G., Rocha, Í.R.C.B., de Sousa, R.R., Costa, F.H.F., 2012. Effects of stocking density on the performance of juvenile pirarucu (*Arapaima gigas*) in cages. Aquaculture 370–371, 96–101. doi:http://dx.doi.org/10.1016/j.aquaculture.2012.09.027

de Souza, R.M., Mourino, J.L., Vieira, F.D., Buglione, C.C., Andreatta, E.R., Seiffert, W.Q., Cerqueira, V.R., 2010. Selection of potential probiotic bacteria to use in fat snook (*Centropomus Parallelus* Poey, 1860) Culture. Bol. Do Inst. Pesca 36, 17–24.

Defoirdt, T., Sorgeloos, P., Bossier, P., 2011. Alternatives to antibiotics for the control of bacterial disease in aquaculture. Curr. Opin. Microbiol. 14, 251–258. doi:http://dx.doi.org/10.1016/j.mib.2011.03.004

Dehler, C.E., Secombes, C.J., Martin, S.A.M., 2017. Environmental and physiological factors shape the gut microbiota of Atlantic salmon parr (*Salmo salar* L.). Aquaculture 467, 149–157. doi:10.1016/j.aquaculture.2016.07.017

Del'Duca, A., Evangelista Cesar, D., Galuppo Diniz, C., Abreu, P.C., 2013. Evaluation of the presence and efficiency of potential probiotic bacteria in the gut of tilapia (*Oreochromis niloticus*) using the fluorescent in situ hybridization technique. Aquaculture 388–391, 115–121. doi:http://dx.doi.org/10.1016/j.aquaculture.2013.01.019

Desai, A.R., Links, M.G., Collins, S.A., Mansfield, G.S., Drew, M.D., Van Kessel, A.G., Hill, J.E., 2012. Effects of plant-based diets on the distal gut microbiome of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 350–353, 134–142. doi:http://dx.doi.org/10.1016/j.aquaculture.2012.04.005

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., Andersen, G.L., 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl. Environ. Microbiol. 72, 5069–5072. doi:10.1128/AEM.03006-05

Dias, M.K.R., Sampaio, L.S., Proietti-Junior, A.A., Yoshioka, E.T.O., Rodrigues, D.P., Rodriguez, A.F.R., Ribeiro, R.A., Faria, F.S.E.D.V., Ozório, R.O.A., Tavares-Dias, M., 2016. Lethal dose and clinical signs of *Aeromonas hydrophila* in *Arapaima gigas* (Arapaimidae), the giant fish from Amazon. Vet. Microbiol. 188, 12–15. doi:http://dx.doi.org/10.1016/j.vetmic.2016.04.001

Dimitroglou, A., Merrifield, D.L., Carnevali, O., Picchietti, S., Avella, M., Daniels,

C., Güroy, D., Davies, S.J., 2011. Microbial manipulations to improve fish health and production – A Mediterranean perspective. Fish Shellfish Immunol. 30, 1–16. doi:http://dx.doi.org/10.1016/j.fsi.2010.08.009

Dosta, M. del C.M., Barrera, T.C., Perrino, F.J.F., Reyes, L.M., Gutiérrez, H.H., Suárez, S.C., 2012. Bacteria with probiotic capabilities isolated from the digestive tract of the ornamental fish *Pterophyllum scalare*, in: Probiotic in Animals. InTech.

Edgar, R.C., 2010. Supplementary Material - Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460–2461. doi:10.1093/bioinformatics/btq461

Ellis, T., North, B., Scott, A.P., Bromage, N.R., Porter, M., Gadd, D., 2002. The relationships between stocking density and welfare in farmed rainbow trout. J. Fish Biol. 61, 493–531. doi:10.1111/j.1095-8649.2002.tb00893.x

Etyemez, M., Balcázar, J.L., 2015. Bacterial community structure in the intestinal ecosystem of rainbow trout (*Oncorhynchus mykiss*) as revealed by pyrosequencing-based analysis of 16S rRNA genes. Res. Vet. Sci. 100, 8–11. doi:https://doi.org/10.1016/j.rvsc.2015.03.026

EFSA - European Food Safety Authority, 2012. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. 10, 1–10. doi:10.2903/j.efsa.2012.2740.

EU, 2005. Regulation 1831/2003/EC on additives for use in animal nutrition, replacing Directive. available in: http://europa.eu/rapid/press-release_IP-05-1687_en.htm 70/524/EEC on additives in feeding-stuffs.

Evans, A.S., 1976. Causation and Disease: The Henle-Koch Postulates Revisited . Yale J. Biol. Med. 49, 175–195.

Falcinelli, S., Picchietti, S., Rodiles, A., Cossignani, L., Merrifield, D.L., Taddei, A.R., Maradonna, F., Olivotto, I., Gioacchini, G., Carnevali, O., 2015. *Lactobacillus rhamnosus* lowers zebrafish lipid content by changing gut microbiota and host transcription of genes involved in lipid metabolism. Sci. Rep. 5, 9336. doi:10.1038/srep09336

Falcinelli, S., Rodiles, A., Hatef, A., Picchietti, S., Cossignani, L., Merrifield, D.L., Unniappan, S., Carnevali, O., 2017. Dietary lipid content reorganizes gut microbiota and probiotic *L. rhamnosus* attenuates obesity and enhances catabolic hormonal milieu in zebrafish. Sci. Rep. 7, 5512. doi:10.1038/s41598-017-05147-w

Falcinelli, S., Rodiles, A., Unniappan, S., Picchietti, S., Gioacchini, G., Merrifield, D.L., Carnevali, O., 2016. Probiotic treatment reduces appetite and glucose level in the zebrafish model. Sci. Rep. 6, 18061. doi:10.1038/srep18061

Farias, T.H.V., Levy-Pereira, N., Alves, L. de O., Dias, D. de C., Tachibana, L., Pilarski, F., Belo, M.A. de A., Ranzani-Paiva, M.J.T., 2016. Probiotic feeding improves the immunity of pacus, *Piaractus mesopotamicus*, during *Aeromonas hydrophila* infection. Anim. Feed Sci. Technol. 211, 137–144. doi:https://doi.org/10.1016/j.anifeedsci.2015.11.004

FAO. 2016. Probiotics in animal nutrition – Production, impact and regulation by Yadav S. Bajagai, Athol V. Klieve, Peter J. Dart and Wayne L. Bryden. Editor

Harinder P.S. Makkar. FAO Animal Production and Health Paper No. 179. Rome.

FAO. 2017 - Fisheries and Aquaculture Information and Statistics Branch - 20/12/2017.

FAO. 2017a. Regional review on status and trends in aquaculture development in Latin America and the Caribbean – 2015, by Carlos Wurmann G. FAO Fisheries and Aquaculture Circular No. 1135/3. Rome, Italy.

Fečkaninová, A., Koščová, J., Mudroňová, D., Popelka, P., Toropilová, J., 2017. The use of probiotic bacteria against *Aeromonas* infections in salmonid aquaculture. Aquaculture 469, 1–8. doi:https://doi.org/10.1016/j.aquaculture.2016.11.042

Ferguson, R.M., Merrifield, D.L., Harper, G.M., Rawling, M.D., Mustafa, S., Picchietti, S., Balcazar, J.L., Davies, S.J., 2010. The effect of *Pediococcus acidilactici* on the gut microbiota and immune status of on-growing red tilapia (*Oreochromis niloticus*). J. Appl. Microbiol. 109, 851–862. doi:10.1111/j.1365-2672.2010.04713.x

Finegold, S.M., Vaisanen, M.-L., Molitoris, D.R., Tomzynski, T.J., Song, Y., Liu, C., Collins, M.D., Lawson, P.A., 2003. *Cetobacterium somerae* sp. nov. from Human Feces and Emended Description of the Genus *Cetobacterium*. Syst. Appl. Microbiol. 26, 177–181. doi:https://doi.org/10.1078/072320203322346010

Foey, A., Picchietti, S., 2014. Immune Defences of Teleost Fish, in: Aquaculture Nutrition. John Wiley & Sons, Ltd, pp. 14–52. doi:10.1002/9781118897263.ch2

Forberg, T., Sjulstad, E.B., Bakke, I., Olsen, Y., Hagiwara, A., Sakakura, Y., Vadstein, O., 2016. Correlation between microbiota and growth in Mangrove Killifish (*Kryptolebias marmoratus*) and Atlantic cod (*Gadus morhua*). Sci. Rep. 6, 21192.

Fracalossi, D.M., Allen, M.E., Yuyama, L.K., Oftedal, O.T., 2001. Ascorbic acid biosynthesis in Amazonian fishes. Aquaculture 192, 321–332. doi:https://doi.org/10.1016/S0044-8486(00)00455-5

Fryer, J.L., Sanders, J.E., 1981. Bacterial Kidney Disease of Salmonid Fish. Annu. Rev. Microbiol. 35, 273–298. doi:10.1146/annurev.mi.35.100181.001421

Gajardo, K., Jaramillo-Torres, A., Kortner, T.M., Merrifield, D.L., Tinsley, J., Bakke, A.M., Krogdahl, Å., 2017. Alternative protein sources in the diet modulate microbiota and functionality in the distal intestine of Atlantic salmon (*Salmo salar*). Appl. Environ. Microbiol. 83. doi:10.1128/AEM.02615-16

Gajardo, K., Rodiles, A., Kortner, T.M., Krogdahl, Å., Bakke, A.M., Merrifield, D.L., Sørum, H., 2016. A high-resolution map of the gut microbiota in Atlantic salmon (*Salmo salar*): A basis for comparative gut microbial research. Sci. Rep. 6, 30893.

Gallani, S.U., Sebastião, F. de A., Valladão, G.M.R., Boaratti, A.Z., Pilarski, F., 2016. Pathogenesis of mixed infection by *Spironucleus* sp. and *Citrobacter freundii* in freshwater angelfish *Pterophyllum scalare*. Microb. Pathog. 100, 119–123. doi:http://dx.doi.org/10.1016/j.micpath.2016.09.002

García de La Banda, I., Lobo, C., León-Rubio, J.M., Tapia-Paniagua, S., Balebona, M.C., Moriñigo, M.A., Moreno-Ventas, X., Lucas, L.M., Linares, F., Arce, F., Arijo, S., 2010. Influence of two closely related probiotics on juvenile

Senegalese sole (*Solea senegalensis*, Kaup 1858) performance and protection against *Photobacterium damselae* subsp. *piscicida*. Aquaculture 306, 281–288. doi:http://dx.doi.org/10.1016/j.aquaculture.2010.05.008

Gatesoupe, F.J., 1999. The use of probiotics in aquaculture. Aquaculture 180, 147–165. doi:Doi 10.1016/S0044-8486(99)00187-8

Gauthier, D.T., 2015. Bacterial zoonoses of fishes: A review and appraisal of evidence for linkages between fish and human infections. Vet. J. 203, 27–35. doi:http://dx.doi.org/10.1016/j.tvjl.2014.10.028

Geraylou, Z., Souffreau, C., Rurangwa, E., De Meester, L., Courtin, C.M., Delcour, J.A., Buyse, J., Ollevier, F., 2013. Effects of dietary arabinoxylan-oligosaccharides (AXOS) and endogenous probiotics on the growth performance, non-specific immunity and gut microbiota of juvenile Siberian sturgeon (*Acipenser baerii*). Fish Shellfish Immunol. 35, 766–775. doi:http://dx.doi.org/10.1016/j.fsi.2013.06.014

Ghanbari, M., Kneifel, W., Domig, K.J., 2015. A new view of the fish gut microbiome: Advances from next-generation sequencing. Aquaculture 448, 464–475. doi:http://dx.doi.org/10.1016/j.aquaculture.2015.06.033

Gisbert, E., Castillo, M., Skalli, A., Andree, K.B., Badiola, I., 2013. *Bacillus cereus* var. *toyoi* promotes growth, affects the histological organization and microbiota of the intestinal mucosa in rainbow trout fingerlings1. J. Anim. Sci. 91, 2766–2774. doi:10.2527/jas.2012-5414

Givens, C.E., Ransom, B., Bano, N., T, H.J., 2015. Comparison of the gut microbiomes of 12 bony fish and 3 shark species. Mar. Ecol. Prog. Ser. 518, 209–223.

Gomez, G.D., Balcazar, J.L., 2008. A review on the interactions between gut microbiota and innate immunity of fish. Fems Immunol. Med. Microbiol. 52, 145–154. doi:DOI 10.1111/j.1574-695X.2007.00343.x

Gonçalves, A.T., Valenzuela-Muñoz, V., Gallardo-Escárate, C., 2017. Intestinal transcriptome modulation by functional diets in rainbow trout: A high-throughput sequencing appraisal to highlight GALT immunomodulation. Fish Shellfish Immunol. 64, 325–338. doi:https://doi.org/10.1016/j.fsi.2017.03.022

Gonzalez, R.J., Brauner, C.J., Wang, Y.X., Richards, J.G., Patrick, M.L., Xi, W., Matey, V., Val, A.L., 2010. Impact of ontogenetic changes in branchial morphology on gill function in *Arapaima gigas*. Physiol Biochem Zool 83, 322–332. doi:10.1086/648568

Gopalakannan, A., Arul, V., 2011. Inhibitory activity of probiotic *Enterococcus faecium* MC13 against *Aeromonas hydrophila* confers protection against hemorrhagic septicemia in common carp *Cyprinus carpio*. Aquac. Int. 19, 973–985.

Hagi, T., Tanaka, D., Iwamura, Y., Hoshino, T., 2004. Diversity and seasonal changes in lactic acid bacteria in the intestinal tract of cultured freshwater fish. Aquaculture 234, 335–346. doi:http://dx.doi.org/10.1016/j.aquaculture.2004.01.018

Harikrishnan, R., Balasundaram, C., Heo, M.-S., 2010a. *Lactobacillus sakei* BK19 enriched diet enhances the immunity status and disease resistance to streptococcosis infection in kelp grouper, *Epinephelus bruneus*. Fish Shellfish

Immunol. 29, 1037–43. doi:10.1016/j.fsi.2010.08.017

Harikrishnan, R., Balasundaram, C., Heo, M.-S., 2010b. Effect of probiotics enriched diet on *Paralichthys olivaceus* infected with lymphocystis disease virus (LCDV). Fish Shellfish Immunol. 29, 868–74. doi:10.1016/j.fsi.2010.07.031

Hatha, M., Vivekanandhan, A.A., Julie Joice, G., Christol, 2005. Antibiotic resistance pattern of motile aeromonads from farm raised fresh water fish. Int. J. Food Microbiol. 98, 131–134. doi:https://doi.org/10.1016/j.ijfoodmicro.2004.05.017

He, S., Zhang, Y., Xu, L., Yang, Y., Marubashi, T., Zhou, Z., Yao, B., 2013. Effects of dietary *Bacillus subtilis* C-3102 on the production, intestinal cytokine expression and autochthonous bacteria of hybrid tilapia *Oreochromis niloticus* ♀ × Oreochromis aureus ♂. Aquaculture 412–413, 125–130. doi:http://dx.doi.org/10.1016/j.aquaculture.2013.06.028

Heo, W.S., Kim, Y.R., Kim, E.Y., Bai, S.C., Kong, I.S., 2013. Effects of dietary probiotic, *Lactococcus lactis* subsp *lactis* I2, supplementation on the growth and immune response of olive flounder (*Paralichthys olivaceus*). Aquaculture 376, 20–24. doi:DOI 10.1016/j.aquaculture.2012.11.009

Hjelm, M., Bergh, Ø., Riaza, A., Nielsen, J., Melchiorsen, J., Jensen, S., Duncan, H., Ahrens, P., Birkbeck, H., Gram, L., 2004. Selection and Identification of Autochthonous Potential Probiotic Bacteria from Turbot Larvae (*Scophthalmus maximus*) Rearing Units. Syst. Appl. Microbiol. 27, 360–371. doi:http://dx.doi.org/10.1078/0723-2020-00256

Hovda, M.B., Fontanillas, R., McGurk, C., Obach, A., Rosnes, J.T., 2012. Seasonal variations in the intestinal microbiota of farmed Atlantic salmon (*Salmo salar* L.). Aquac. Res. 43, 154–159. doi:10.1111/j.1365-2109.2011.02805.x

Hummel, A., Holzapfel, W.H., Franz, C.M., 2007. Characterisation and transfer of antibiotic resistance genes from enterococci isolated from food. Syst. Appl. Microbiol. 30, 1–7. doi:10.1016/j.syapm.2006.02.004

Inglis, V., Roberts, R.J., Bromage, N.R., 1993. Bacterial Diseases of Fish. Wiley.

Irianto, A., Austin, B., 2002a. Probiotics in aquaculture. J. Fish Dis. 25, 633–642. doi:10.1046/j.1365-2761.2002.00422.x

Irianto, A., Austin, B., 2002b. Use of probiotics to control furunculosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish Dis. 25, 333–342. doi:10.1046/j.1365-2761.2002.00375.x

Jang, Y.-H., Subramanian, D., Heo, M.-S., 2014. Efficacy of formalin-killed *Pseudomonas anguilliseptica* vaccine on immune gene expression and protection in farmed olive flounder, *Paralichthys olivaceus*. Vaccine 32, 1808–1813. doi:http://dx.doi.org/10.1016/j.vaccine.2014.01.088

Jatoba, A., Vieira, F.D., Buglione-Neto, C.C., Mourino, J.L.P., Silva, B.C., Seiftter, W.Q., Andreatta, E.R., 2011. Diet supplemented with probiotic for Nile tilapia in polyculture system with marine shrimp. Fish Physiol. Biochem. 37, 725–732. doi:DOI 10.1007/s10695-011-9472-5

Jeremić, S., Jakić-Dimić, D., Veljović, L.J., 2003. *Citrobacter freundii* as a cause of disease in fish. Acta Vet. Brno. 53, 399–410.

Jesus, G.F.A., Vieira, F.D.N., Silva, B.C., Junior, M.M.D.S., Ushizima, T.T., Schmidt, E.C., Bouzon, Z.L., Pereira, S.A., Pereira, G. V, Martins, M.L.,

Mouriño, J.L.P., 2016. Probiotic bacteria may prevent haemorrhagic septicaemia by maturing intestinal host defences in Brazilian native surubins. Aquac. Nutr. n/a-n/a. doi:10.1111/anu.12416

Ju, F., Zhang, T., 2015. 16S rRNA gene high-throughput sequencing data mining of microbial diversity and interactions. Appl. Microbiol. Biotechnol. 99, 4119–29. doi:10.1007/s00253-015-6536-y

Karunasagar, I., Karunasagar, I., Pai, R., 1992. Systemic *Citrobacter freundii* infection in common carp, *Cyprinus carpio* L., fingerlings. J. Fish Dis. 15, 95–98. doi:10.1111/j.1365-2761.1992.tb00642.x

Kennedy, S.B., Tucker Jr, J.W., Neidig, C.L., Vermeer, G.K., Cooper, V.R., Jarrell, J.L., Sennett, D.G., 1998. Bacterial management strategies for stock enhancement of warmwater marine fish: a case study with common snook (*Centropomus undecimalis*). Bull. Mar. Sci. 62, 573–588.

Kibenge, F.S., Munir, K., Kibenge, M.J., Joseph, T., Moneke, E., 2004. Infectious salmon anemia virus: causative agent, pathogenesis and immunity. Anim Heal. Res Rev 5, 65–78. doi:S1466252304000052 [pii]

Kim, D.-H., Austin, B., 2006. Innate immune responses in rainbow trout (*Oncorhynchus mykiss*, Walbaum) induced by probiotics. Fish Shellfish Immunol. 21, 513–524. doi:https://doi.org/10.1016/j.fsi.2006.02.007

Kim, D., Beck, B.R., Heo, S.-B., Kim, J., Kim, H.D., Lee, S.-M., Kim, Y., Oh, S.Y., Lee, K., Do, H., Lee, K., Holzapfel, W.H., Song, S.K., 2013. *Lactococcus lactis* BFE920 activates the innate immune system of olive flounder (*Paralichthys olivaceus*), resulting in protection against *Streptococcus iniae* infection and enhancing feed efficiency and weight gain in large-scale field studies. Fish Shellfish Immunol. 35, 1585–1590. doi:http://dx.doi.org/10.1016/j.fsi.2013.09.008

Kumar, R., Mukherjee, S.C., Ranjan, R., Nayak, S.K., 2008. Enhanced innate immune parameters in *Labeo rohita* (Ham.) following oral administration of *Bacillus subtilis*. Fish Shellfish Immunol. 24, 168–172. doi:https://doi.org/10.1016/j.fsi.2007.10.008

Laing, K.J., Hansen, J.D., 2011. Fish T cells: Recent advances through genomics. Dev. Comp. Immunol. 35, 1282–1295. doi:http://dx.doi.org/10.1016/j.dci.2011.03.004

Larsen, a. M., Mohammed, H.H., Arias, C.R., 2014. Characterization of the gut microbiota of three commercially valuable warmwater fish species. J. Appl. Microbiol. 116, 1396–1404. doi:10.1111/jam.12475

Lauková, A., 2011. Potential Applications of Probiotic, Bacteriocin-Producing Enterococci and Their Bacteriocins, in: Lactic Acid Bacteria. CRC Press, pp. 39–61. doi:10.1201/b11503-4

Lauzon, H., Ringo, E., 2011. Prevalence and Application of Lactic Acid Bacteria in Aquatic Environments, in: Lactic Acid Bacteria. CRC Press, pp. 593–631. doi:10.1201/b11503-29

Lauzon, H.L., Dimitroglou, A., Merrifield, D.L., Ringø, E., Davies, S.J., 2014. Probiotics and Prebiotics: Concepts, Definitions and History, in: Aquaculture Nutrition. John Wiley & Sons, Ltd, pp. 169–184. doi:10.1002/9781118897263.ch7

Lauzon, H.L., Gudmundsdottir, S., Steinarsson, A., Oddgeirsson, M., Martinsdottir, E., Gudmundsdottir, B.K., 2010. Impact of probiotic intervention on microbial load and performance of Atlantic cod (*Gadus morhua* L.) juveniles. Aquaculture 310, 139–144. doi:https://doi.org/10.1016/j.aquaculture.2010.10.017

Lee, S., Katya, K., Park, Y., Won, S., Seong, M., hamidoghli, A., Bai, S.C., 2017. Comparative evaluation of dietary probiotics *Bacillus subtilis* WB60 and *Lactobacillus plantarum* KCTC3928 on the growth performance, immunological parameters, gut morphology and disease resistance in Japanese eel, *Anguilla japonica*. Fish Shellfish Immunol. 61, 201–210. doi:https://doi.org/10.1016/j.fsi.2016.12.035

Li, J., Ni, J., Li, J., Wang, C., Li, X., Wu, S., Zhang, T., Yu, Y., Yan, Q., 2014. Comparative study on gastrointestinal microbiota of eight fish species with different feeding habits. J. Appl. Microbiol. n/a-n/a. doi:10.1111/jam.12663

Li, T., Long, M., Gatesoupe, F.-J., Zhang, Q., Li, A., Gong, X., 2015. Comparative Analysis of the Intestinal Bacterial Communities in Different Species of Carp by Pyrosequencing. Microb. Ecol. 69, 25–36. doi:10.1007/s00248-014-0480-8

Lin, H.-L., Shiu, Y.-L., Chiu, C.-S., Huang, S.-L., Liu, C.-H., 2017. Screening probiotic candidates for a mixture of probiotics to enhance the growth performance, immunity, and disease resistance of Asian seabass, *Lates calcarifer* (Bloch), against *Aeromonas hydrophila*. Fish Shellfish Immunol. 60, 474–482. doi:https://doi.org/10.1016/j.fsi.2016.11.026

Llewellyn, M.S., Boutin, S., Hoseinifar, S.H., Derome, N., 2014. Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. Front. Microbiol. 5, 207. doi:10.3389/fmicb.2014.00207

Lü, A., Hu, X., Zheng, L., Zhu, A., Cao, C., Jiang, J., 2011. Isolation and characterization of *Citrobacter* spp. from the intestine of grass carp *Ctenopharyngodon idellus*. Aquaculture 313, 156–160. doi:http://dx.doi.org/10.1016/j.aquaculture.2011.01.018

Lyons, P.P., Turnbull, J.F., Dawson, K.A., Crumlish, M., 2017. Exploring the microbial diversity of the distal intestinal lumen and mucosa of farmed rainbow trout *Oncorhynchus mykiss* (Walbaum) using next generation sequencing (NGS). Aquac. Res. 48, 77–91. doi:10.1111/are.12863

Magnadottir, B., 2010. Immunological control of fish diseases. Mar Biotechnol 12, 361–379. doi:10.1007/s10126-010-9279-x

Mathur, S., Singh, R., 2005. Antibiotic resistance in food lactic acid bacteria--a review. Int. J. Food Microbiol. 105, 281–295. doi:10.1016/j.ijfoodmicro.2005.03.008

Maturin, L., Peeler, J.T., 2001. Bacteriological Analytical Manual Chapter 3. Aerobic Plate Count. 2001.

Mehrabi, Z., Firouzbakhsh, F., Jafarpour, A., 2012. Effects of dietary supplementation of synbiotic on growth performance, serum biochemical parameters and carcass composition in rainbow trout (*Oncorhynchus mykiss*) fingerlings. J. Anim. Physiol. Anim. Nutr. (Berl). 96, 474–481. doi:DOI 10.1111/j.1439-0396.2011.01167.x

Merrifield, D., Ringo, E., 2014. Aquaculture Nutrition Gut Health, Probiotics, and

Prebiotics. Chichester, West Sussex : John Wiley & Sons, Inc.

Merrifield, D.L., Balcázar, J.L., Daniels, C., Zhou, Z., Carnevali, O., Sun, Y.-Z., Hoseinifar, S.H., Ringø, E., 2014. Indigenous Lactic Acid Bacteria in Fish and Crustaceans, in: Aquaculture Nutrition. John Wiley & Sons, Ltd, pp. 128–168. doi:10.1002/9781118897263.ch6

Merrifield, D.L., Bradley, G., Baker, R.T.M., Davies, S.J., 2010. Probiotic applications for rainbow trout (*Oncorhynchus mykiss* Walbaum) II. Effects on growth performance, feed utilization, intestinal microbiota and related health criteria postantibiotic treatment. Aquac. Nutr. 16, 496–503. doi:DOI 10.1111/j.1365-2095.2009.00688.x

Merrifield, D.L., Carnevali, O., 2014. Probiotic Modulation of the Gut Microbiota of Fish, in: Aquaculture Nutrition. John Wiley & Sons, Ltd, pp. 185–222. doi:10.1002/9781118897263.ch8

Merrifield, D.L., Dimitroglou, A., Foey, A., Davies, S.J., Baker, R.T.M., Bøgwald, J., Castex, M., Ringø, E., 2010. The current status and future focus of probiotic and prebiotic applications for salmonids. Aquaculture 302, 1–18. doi:http://dx.doi.org/10.1016/j.aquaculture.2010.02.007

Miruka, D.O., Ochieng, J.O., Were, J.W., Waindi, E.N., 2013. Microbial assessment of selected earthen fish ponds in western Kenya. Ecohydrol. Hydrobiol. 13, 261–266. doi:http://dx.doi.org/10.1016/j.ecohyd.2013.10.005

Mohapatra, S., Chakraborty, T., Prusty, A.K., Das, P., Paniprasad, K., Mohanta, K.N., 2012. Use of different microbial probiotics in the diet of rohu, *Labeo rohita* fingerlings: effects on growth, nutrient digestibility and retention, digestive enzyme activities and intestinal microflora. Aquac. Nutr. 18, 1–11. doi:10.1111/j.1365-2095.2011.00866.x

Mouriño, J.L.P., Pereira, G. do V., Vieira, F. do N., Jatobá, A.B., Ushizima, T.T., Silva, B.C. da, Seiffert, W.Q., Jesus, G.F.A., Martins, M.L., 2016. Isolation of probiotic bacteria from the hybrid South American catfish *Pseudoplatystoma reticulatum*×*Pseudoplatystoma corruscans* (Siluriformes: Pimelodidae): A haematological approach. Aquac. Reports 3, 166–171. doi:https://doi.org/10.1016/j.aqrep.2016.03.001

Mourino, J.L.P., Vieira, F.D., Jatoba, A.B., da Silva, B.C., Jesus, G.F.A., Seiffert, W.Q., Martins, M.L., 2012. Effect of dietary supplementation of inulin and *W. cibaria* on haemato-immunological parameters of hybrid surubim (*Pseudoplatystoma* sp). Aquac. Nutr. 18, 73–80. doi:DOI 10.1111/j.1365-2095.2011.00879.x

Mouriño, J.L.P., Vieira, F.N., Jatobá, A., Silva, B.C., Pereira, G. V, Jesus, G.F.A., Ushizima, T.T., Seiffert, W.Q., Martins, M.L., 2015. Symbiotic supplementation on the hemato-immunological parameters and survival of the hybrid surubim after challenge with *Aeromonas hydrophila*. Aquac. Nutr. doi:10.1111/anu.12390 *in press.*

Munir, M.B., Hashim, R., Chai, Y.H., Marsh, T.L., Nor, S.A.M., 2016. Dietary prebiotics and probiotics influence growth performance, nutrient digestibility and the expression of immune regulatory genes in snakehead (*Channa striata*) fingerlings. Aquaculture 460, 59–68. doi:https://doi.org/10.1016/j.aquaculture.2016.03.041

Munoz-Atienza, E., Gomez-Sala, B., Araujo, C., Campanero, C., del Campo, R., Hernandez, P.E., Herranz, C., Cintas, L.M., 2013. Antimicrobial activity, antibiotic susceptibility and virulence factors of Lactic Acid Bacteria of aquatic origin intended for use as probiotics in aquaculture. BMC Microbiol 13, 15. doi:10.1186/1471-2180-13-15

Nayak, S.K., 2010. Probiotics and immunity: A fish perspective. Fish Shellfish Immunol. 29, 2–14. doi:http://dx.doi.org/10.1016/j.fsi.2010.02.017

Newaj-Fyzul, A., Al-Harbi, A.H., Austin, B., 2014. Review: Developments in the use of probiotics for disease control in aquaculture. Aquaculture 431, 1–11. doi:http://dx.doi.org/10.1016/j.aquaculture.2013.08.026

Ng, L.K., Martin, I., Alfa, M., Mulvey, M., 2001. Multiplex PCR for the detection of tetracycline resistant genes. Mol Cell Probes 15, 209–215. doi:10.1006/mcpr.2001.0363

Nguyen, T.L., Park, C.-I., Kim, D.-H., 2017. Improved growth rate and disease resistance in olive flounder, *Paralichthys olivaceus*, by probiotic *Lactococcus lactis* WFLU12 isolated from wild marine fish. Aquaculture 471, 113–120. doi:https://doi.org/10.1016/j.aquaculture.2017.01.008

Nikoskelainen, S., Ouwehand, A.C., Bylund, G., Salminen, S., Lilius, E.M., 2003. Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotic bacteria (*Lactobacillus rhamnosus*). Fish Shellfish Immunol 15, 443–452.

Noffs, A.P., Tachibana, L., Santos, A.A., Ranzani-Paiva, M.J.T., 2015. Common snook fed in alternate and continuous regimens with diet supplemented with *Bacillus subtilis* probiotic. Pesqui. Agropecuária Bras. 50, 267–272.

Ono, E.A., Halverson, M.R., Kubitza, F., 2004. Pirarucu, o gigante esquecido. Panor. da Aquicultura 14, 14–25.

Pádua, S.B. de, Marques, D.P., Sebastião, F.A., Pilarski, F., Martins, M.L., Ishikawa, M.M., 2014. Isolation, Characterization and Pathology of *Citrobacter freundii* Infection in Native Brazilian Catfish *Pseudoplatystoma*. Brazilian J. Vet. Pathol. Brazilian J. Vet. Pathol. 7, 151 -157.

Panigrahi, A., Kiron, V., Kobayashi, T., Puangkaew, J., Satoh, S., Sugita, H., 2004. Immune responses in rainbow trout *Oncorhynchus mykiss* induced by a potential probiotic bacteria *Lactobacillus rhamnosus* JCM 1136. Vet. Immunol. Immunopathol. 102, 379–388. doi:DOI 10.1016/j.vetimm.2004.08.006

Panigrahi, A., Kiron, V., Satoh, S., Hirono, I., Kobayshi, T., Sugita, H., Puangkaew, J., Aoki, T., 2007. Immune modulation and expression of cytokine genes in rainbow trout *Oncorhynchus mykiss* upon probiotic feeding. Dev. Comp. Immunol. 31, 372–382. doi:DOI 10.1016/j.dci.2006.07.004

Parma, L., Candela, M., Soverini, M., Turroni, S., Consolandi, C., Brigidi, P., Mandrioli, L., Sirri, R., Fontanillas, R., Gatta, P.P., Bonaldo, A., 2016. Next-generation sequencing characterization of the gut bacterial community of gilthead sea bream (*Sparus aurata*, L.) fed low fishmeal based diets with increasing soybean meal levels. Anim. Feed Sci. Technol. 222, 204–216. doi:https://doi.org/10.1016/j.anifeedsci.2016.10.022

Pereira-Filho, M., Cavero, B.A.S., Roubach, R., Ituassú, D.R., Gandra, A.L., Crescêncio, R., 2003. Cultivo do Pirarucu (*Arapaima gigas*) em viveiro escavado

. Acta Amaz. .

Pereira, G. do V., Silva, B.C. da, Vieira, F. do N., Seiffert, W.Q., Ushizima, T.T., Mouriño, J.L.P., Martins, M.L., 2015. Vaccination strategies with oral booster for surubim hybrid (*Pseudoplatystoma corruscans* × *P. reticulatum*) against haemorrhagic septicaemia. Aquac. Res. 46, 1831–1841. doi:10.1111/are.12339

Pereira, G. V., Cunha, D.G., Pedreira Mourino, J.L., Rodiles, A., Jaramillo-Torres, A., Merrifield, D.L., 2017. Characterization of microbiota in *Arapaima gigas* intestine and isolation of potential probiotic bacteria. J. Appl. Microbiol. doi:10.1111/jam.13572

Picchietti, S., Fausto, A.M., Randelli, E., Carnevali, O., Taddei, A.R., Buonocore, F., Scapigliati, G., Abelli, L., 2009. Early treatment with *Lactobacillus delbrueckii* strain induces an increase in intestinal T-cells and granulocytes and modulates immune-related genes of larval *Dicentrarchus labrax* (L.). Fish Shellfish Immunol. 26, 368–376. doi:http://dx.doi.org/10.1016/j.fsi.2008.10.008

Portella, M.C., Jomori, R.K., Leitão, N.J., Menossi, O.C.C., Freitas, T.M., Kojima, J.T., Lopes, T.S., Clavijo-Ayala, J.A., Carneiro, D.J., 2014. Larval development of indigenous South American freshwater fish species, with particular reference to pacu (*Piaractus mesopotamicus*): A review. Aquaculture 432, 402–417. doi:http://dx.doi.org/10.1016/j.aquaculture.2014.04.032

Ramos, M.A., Weber, B., Gonçalves, J.F., Santos, G.A., Rema, P., Ozório, R.O.A., 2013. Dietary probiotic supplementation modulated gut microbiota and improved growth of juvenile rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 166, 302–307. doi:http://dx.doi.org/10.1016/j.cbpa.2013.06.025

Rasmussen, R., 2001. Quantification on the LightCycler. Rapid cycle real-time PCR, methods Appl. 1, 21–34.

Reite, O.B., Evensen, Ø., 2006. Inflammatory cells of teleostean fish: A review focusing on mast cells/eosinophilic granule cells and rodlet cells. Fish Shellfish Immunol. 20, 192–208. doi:http://dx.doi.org/10.1016/j.fsi.2005.01.012

Ridha, M.T., Azad, I.S., 2012. Preliminary evaluation of growth performance and immune response of Nile tilapia *Oreochromis niloticus* supplemented with two putative probiotic bacteria. Aquac. Res. 43, 843–852. doi:10.1111/j.1365-2109.2011.02899.x

Ringø, E., Gatesoupe, F.J., 1998. Lactic acid bacteria in fish: a review. Aquaculture 160, 177–203. doi:Doi 10.1016/S0044-8486(97)00299-8

Ringø, E., Lovmo, L., Kristiansen, M., Bakken, Y., Salinas, I., Myklebust, R., Olsen, R.E., Mayhew, T.M., 2010. Lactic acid bacteria vs. pathogens in the gastrointestinal tract of fish: a review. Aquac. Res. 41, 451–467. doi:DOI 10.1111/j.1365-2109.2009.02339.x

Ringø, E., Zhou, Z., Vecino, J.L.G., Wadsworth, S., Romero, J., Krogdahl, Å., Olsen, R.E., Dimitroglou, A., Foey, A., Davies, S., Owen, M., Lauzon, H.L., Martinsen, L.L., De Schryver, P., Bossier, P., Sperstad, S., Merrifield, D.L., 2016. Effect of dietary components on the gut microbiota of aquatic animals. A never-ending story? Aquac. Nutr. 22, 219–282. doi:10.1111/anu.12346

Rodrigues, J.B., Leitão, N.J., Chaves, K.S., Gigante, M.L., Portella, M.C., Grosso, C.R.F., 2014. High protein microparticles produced by ionic gelation containing

*Lactobacillus acidophilus* for feeding pacu larvae. Food Res. Int. 63, 25–32. doi:https://doi.org/10.1016/j.foodres.2014.02.005

Rodriguez-Estrada, U., Satoh, S., Haga, Y., Fushimi, H., Sweetman, J., 2013. Effects of Inactivated *Enterococcus faecalis* and Mannan Oligosaccharide and Their Combination on Growth, Immunity, and Disease Protection in Rainbow Trout. N. Am. J. Aquac. 75, 416–428. doi:Doi 10.1080/15222055.2013.799620

Roeselers, G., Mittge, E.K., Stephens, W.Z., Parichy, D.M., Cavanaugh, C.M., Guillemin, K., Rawls, J.F., 2011. Evidence for a core gut microbiota in the zebrafish. ISME J 5, 1595–1608. doi:http://www.nature.com/ismej/journal/v5/n10/suppinfo/ismej201138s1.html

Román, L., Real, F., Sorroza, L., Padilla, D., Acosta, B., Grasso, V., Bravo, J., Acosta, F., 2012. The *in vitro* effect of probiotic *Vagococcus fluvialis* on the innate immune parameters of *Sparus aurata* and *Dicentrarchus labrax*. Fish Shellfish Immunol. 33, 1071–1075. doi:https://doi.org/10.1016/j.fsi.2012.06.028

Rombout, J.H., Abelli, L., Picchietti, S., Scapigliati, G., Kiron, V., 2011. Teleost intestinal immunology. Fish Shellfish Immunol 31, 616–626. doi:S1050-4648(10)00288-3 [pii]10.1016/j.fsi.2010.09.001

Romero, J., Feijoo, C.G., Navarrete, P., 2012. Antibiotics in Aquaculture – Use, Abuse and Alternatives, in: Carvalho, E.D., David, G.S., Silva, R.J. (Eds.), Health and Environment in Aquaculture. InTech, pp. 6–198. doi:0.5772/28157

Romero, J., Ringø, E., Merrifield, D.L., 2014. The Gut Microbiota of Fish, in: Aquaculture Nutrition. John Wiley & Sons, Ltd, pp. 75–100. doi:10.1002/9781118897263.ch4

Rosenfeld, G., 1947. Pancromic stain for haematology and clinical cytology. A new combination of the components May-Grünwald and Giemsa in just one formula for rapid staining. Memorias Inst. do Butantan 20, 329–334.

Rurangwa, E., Sipkema, D., Kals, J., ter Veld, M., Forlenza, M., Bacanu, G.M., Smidt, H., Palstra, A.P., 2015. Impact of a novel protein meal on the gastrointestinal microbiota and the host transcriptome of larval zebrafish *Danio rerio*. Front. Physiol. 6, 133. doi:10.3389/fphys.2015.00133

Sáenz de Rodrigáñez, M.A., Díaz-Rosales, P., Chabrillón, M., Smidt, H., Arijo, S., León-Rubio, J.M., Alarcón, F.J., Balebona, M.C., Moriñigo, M.A., Cara, J.B., Moyano, F.J., 2009. Effect of dietary administration of probiotics on growth and intestine functionality of juvenile Senegalese sole (*Solea senegalensis*, Kaup 1858). Aquac. Nutr. 15, 177–185. doi:10.1111/j.1365-2095.2008.00581.x

Saint-Paul, U., 1986. Potential for aquaculture of South American freshwater fishes: A review. Aquaculture 54, 205–240. doi:http://dx.doi.org/10.1016/0044-8486(86)90329-7

Salinas, I., Cuesta, A., Esteban, M.A., Meseguer, J., 2005. Dietary administration of *Lactobacillus delbrueckii* and *Bacillus subtilis*, single or combined, on gilthead seabream cellular innate immune responses. Fish Shellfish Immunol. 19, 67–77. doi:DOI 10.1016/j.fsi.2004.11.007

Salinas, I., Magadán, S., 2017. Omics in fish mucosal immunity. Dev. Comp. Immunol. 75, 99–108. doi:https://doi.org/10.1016/j.dci.2017.02.010

Scapigliati, G., Romano, N., Buonocore, F., Picchietti, S., Baldassini, M.R., Prugnoli,

D., Galice, A., Meloni, S., Secombes, C.J., Mazzini, M., Abelli, L., 2002. The immune system of sea bass, *Dicentrarchus labrax*, reared in aquaculture. Dev. Comp. Immunol. 26, 151–160. doi:S0145305X0100057X [pii]

SEBRAE. 2010. Produção do Pirarucu em Cativeiro. SEBRAE, Porto Velho, 42 pp.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S., Huttenhower, C., 2011. Metagenomic biomarker discovery and explanation. Genome Biol. 12, R60–R60. doi:10.1186/gb-2011-12-6-r60

Sharma, P., Tomar, S.K., Goswami, P., Sangwan, V., Singh, R., 2014. Antibiotic resistance among commercially available probiotics. Food Res. Int. 57, 176–195. doi:DOI 10.1016/j.foodres.2014.01.025

Shelby, R.A., Lim, C., Yildirim-Aksoy, M., Klesius, P.H., 2007. Effects of Probiotic Bacteria as Dietary Supplements on Growth and Disease Resistance in Young Channel Catfish, *Ictalurus punctatus* (Rafinesque). J. Appl. Aquac. 19, 81–91. doi:10.1300/J028v19n01_a

Shinn, A., Pratoomyot, J., Bron, J.E., Paladini, G., Brooker, E., Brooker, A.J., 2015. Economic impacts of aquatic parasites on global finfish production. Glob. Aquac. Advocate 82–84.

Sloman, K.A., Motherwell, G., O'Connor, K.I., Taylor, A.C., 2000. The effect of social stress on the Standard Metabolic Rate (SMR) of brown trout, Salmo trutta. Fish Physiol. Biochem. 23, 49–53. doi:10.1023/A:1007855100185

Souza, D.M. de, Martins, G.B., Piedras, S.R.N., Pouey, J.L.O.F., Robaldo, R.B., Leite, F.P.L., 2012. Probiotic actions of Bacillus cereus var. toyoi and *Saccharomyces boulardii* in silver catfish (*Rhamdia quelen*) larvae culture . Rev. Bras. Zootec. .

Standen, B.T., Rawling, M.D., Davies, S.J., Castex, M., Foey, A., Gioacchini, G., Carnevali, O., Merrifield, D.L., 2013a. Probiotic *Pediococcus acidilactici* modulates both localised intestinal- and peripheral-immunity in tilapia (*Oreochromis niloticus*). Fish Shellfish Immunol. 35, 1097–1104. doi:http://dx.doi.org/10.1016/j.fsi.2013.07.018

Standen, B.T., Rodiles, A., Peggs, D.L., Davies, S.J., Santos, G.A., Merrifield, D.L., 2015. Modulation of the intestinal microbiota and morphology of tilapia, *Oreochromis niloticus*, following the application of a multi-species probiotic. Appl. Microbiol. Biotechnol. 99, 8403–8417. doi:10.1007/s00253-015-6702-2

Stephens, W.Z., Burns, A.R., Stagaman, K., Wong, S., Rawls, J.F., Guillemin, K., Bohannan, B.J.M., 2016. The composition of the zebrafish intestinal microbial community varies across development. ISME J. 10, 644–654. doi:10.1038/ismej.2015.140

Sugita, H., Shibuya, K., Shimooka, H., Deguchi, Y., 1996. Antibacterial abilities of intestinal bacteria in freshwater cultured fish. Aquaculture 145, 195–203. doi:http://dx.doi.org/10.1016/S0044-8486(96)01319-1

Sun, Y.Z., Yang, H.L., Huang, K.P., Ye, J.D., Zhang, C.X., 2013. Application of autochthonous *Bacillus* bioencapsulated in copepod to grouper *Epinephelus coioides* larvae. Aquaculture 392, 44–50. doi:DOI 10.1016/j.aquaculture.2013.01.037

Sutcliffe, J., Grebe, T., Tait-Kamradt, A., Wondrack, L., 1996. Detection of

erythromycin-resistant determinants by PCR. Antimicrob Agents Chemother 40, 2562–2566.

Tarnecki, A.M., Burgos, F.A., Ray, C.L., Arias, C.R., 2017. Fish intestinal microbiome: diversity and symbiosis unravelled by metagenomics. J. Appl. Microbiol. 123, 2–17. doi:10.1111/jam.13415

Tavares-Dias, M., Barcellos, J.F.M., Marcon, J.L., Menezes, G.C., Ono, E.A., Affonso, E.G., 2007. Hematological and biochemical parameters for the pirarucu *Arapaima gigas* Schinz, 1822 (Osteoglossiformes, Arapaimatidae) in net cage culture. Electron. J. Ichthyol. 2, 61–68.

Tavares-Dias, M., Martins, M.L., 2017. An overall estimation of losses caused by diseases in the Brazilian fish farms. J. Parasit. Dis. doi:10.1007/s12639-017-0938-y

Thanigaivel, S., Vijayakumar, S., Gopinath, S., Mukherjee, A., Chandrasekaran, N., Thomas, J., 2015. *In vivo* and *in vitro* antimicrobial activity of Azadirachta indica (Lin) against *Citrobacter freundii* isolated from naturally infected Tilapia (*Oreochromis mossambicus*). Aquaculture 437, 252–255. doi:http://dx.doi.org/10.1016/j.aquaculture.2014.12.008

Toranzo, A.E., Magarinos, B., Romalde, J.L., 2005. A review of the main bacterial fish diseases in mariculture systems. Aquaculture 246, 37–61. doi:DOI 10.1016/j.aquaculture.2005.01.002

Tsuchiya, C., Sakata, T., Sugita, H., 2008. Novel ecological niche of *Cetobacterium somerae*, an anaerobic bacterium in the intestinal tracts of freshwater fish. Lett. Appl. Microbiol. 46, 43–48. doi:10.1111/j.1472-765X.2007.02258.x

Ullah, A., Zuberi, A., Ahmad, M., Bashir Shah, A., Younus, N., Ullah, S., Khattak, M.N.K., 2018. Dietary administration of the commercially available probiotics enhanced the survival, growth, and innate immune responses in Mori (*Cirrhinus mrigala*) in a natural earthen polyculture system. Fish Shellfish Immunol. 72, 266–272. doi:https://doi.org/10.1016/j.fsi.2017.10.056

Verschuere, L., Rombaut, G., Sorgeloos, P., Verstraete, W., 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiol. Mol. Biol. Rev. 64, 655–671.

Vieira, F. do N., Pedrotti, F.S., Buglione Neto, C.C., Mouriño, J.L.P., Beltrame, E., Martins, M.L., Ramirez, C., Arana, L.A.V., 2007. Lactic-acid bacteria increase the survival of marine shrimp, *Litopenaeus vannamei*, after infection with *Vibrio harveyi*. Brazilian J. Oceanogr. 55, 251–255.

Vine, N.G., Leukes, W.D., Kaiser, H., 2006. Probiotics in marine larviculture. FEMS Microbiol. Rev. 30, 404–427.

Vlková, E., Kalous, L., Bunešová, V., Rylková, K., Světlíková, R., Rada, V., 2012. Occurrence of bifidobacteria and lactobacilli in digestive tract of some freshwater fishes. Biologia (Bratisl). 67, 411–416. doi:10.2478/s11756-012-0017-x

von Wright, A., 2011. Genus *Lactococcus*, in: Lactic Acid Bacteria. CRC Press, pp. 63–76. doi:10.1201/b11503-5

von Wright, A., Axelsson, L., 2011. Lactic Acid Bacteria, in: Lactic Acid Bacteria. CRC Press, pp. 1–16. doi:10.1201/b11503-2

Wackett, L.P., 2016. Lactic acid bacteria. Microb. Biotechnol. 9, 525–526. doi:10.1111/1751-7915.12371

Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. 73, 5261–7. doi:10.1128/AEM.00062-07

Wang, Y.-B., Tian, Z.-Q., Yao, J.-T., Li, W., 2008. Effect of probiotics, *Enteroccus faecium*, on tilapia (*Oreochromis niloticus*) growth performance and immune response. Aquaculture 277, 203–207. doi:http://dx.doi.org/10.1016/j.aquaculture.2008.03.007

Watson, L.C., Stewart, D.J., Teece, M.A., 2013. Trophic ecology of Arapaima in Guyana: giant omnivores in Neotropical floodplains. Neotrop. Ichthyol. 11, 341–349.

Wong, S., Rawls, J.F., 2012. Intestinal microbiota composition in fishes is influenced by host ecology and environment. Mol. Ecol. 21, 3100–3102. doi:10.1111/j.1365-294X.2012.05646.x

Wong, S., Stephens, W.Z., Burns, A.R., Stagaman, K., David, L.A., Bohannan, B.J.M., Guillemin, K., Rawls, J.F., 2015. Ontogenetic Differences in Dietary Fat Influence Microbiota Assembly in the Zebrafish Gut. MBio 6, e00687-15. doi:10.1128/mBio.00687-15

Wong, S., Waldrop, T., Summerfelt, S., Davidson, J., Barrows, F., Kenney, P.B., Welch, T., Wiens, G.D., Snekvik, K., Rawls, J.F., Good, C., 2013. Aquacultured rainbow trout (*Oncorhynchus mykiss*) possess a large core intestinal microbiota that is resistant to variation in diet and rearing density. Appl. Environ. Microbiol. 79, 4974–4984. doi:10.1128/aem.00924-13

WHO guidelines on use of medically important antimicrobials in food-producing animals. Geneva: World Health Organization; 2017. Licence: CC BY-NC-SA 3.0 IGO.

Wu, S., Gao, T., Zheng, Y., Wang, W., Cheng, Y., Wang, G., 2010. Microbial diversity of intestinal contents and mucus in yellow catfish (*Pelteobagrus fulvidraco*). Aquaculture 303, 1–7. doi:https://doi.org/10.1016/j.aquaculture.2009.12.025

Xu, J., Zeng, X., Jiang, N., Zhou, Y., Zeng, L., 2015. *Pseudomonas alcaligenes* infection and mortality in cultured Chinese sturgeon, *Acipenser sinensis*. Aquaculture 446, 37–41. doi:http://dx.doi.org/10.1016/j.aquaculture.2015.04.014

Zhou, Q.-C., Buentello, J.A., Gatlin Iii, D.M., 2010. Effects of dietary prebiotics on growth performance, immune response and intestinal morphology of red drum (*Sciaenops ocellatus*). Aquaculture 309, 253–257. doi:http://dx.doi.org/10.1016/j.aquaculture.2010.09.003

# Appendix

## Apendix: Suplementary data

Table S3b.1: Average of number of dead pirarucu (*Arapaima gigas*) per treatment and final percentage of mortality after 72 hours of infection with *Citrobacter freundii* and *Pseudomonas* sp.

| **Strains** | **Infection dose (CFU.ml$^{-1}$)** | **Hours post infection** | | | | | | **Final mortality (%)** |
|---|---|---|---|---|---|---|---|---|
| | | **12** | **24** | **36** | **48** | **60** | **72** | |
| *Pseudomonas* sp. | 1x10$^8$ | 0 | 0 | 0 | 1 | 1 | 1 | 6.25 |
| | 1x10$^6$ | 0 | 0 | 0 | 0 | 1 | 1 | 6.25 |
| | 1x10$^4$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *C. freundii* | 1x10$^8$ | 6 | 8 | 8 | 8 | 8 | 8 | 50 |
| | 1x10$^6$ | 2 | 4 | 5 | 5 | 5 | 5 | 31.25 |
| | 1x10$^4$ | 0 | 0 | 0 | 1 | 1 | 1 | 6.25 |
| PBS (Control) | - | 0 | 0 | 2 | 2 | 2 | 2 | 12.5 |

Table S3b.2: Calculations for Probit (P) and Logit (P) Linear Model after 72 h of infection with *Citrobacter freundii* and compared with Control (0).

| | | | | Mortality proportion | Corrected proportion | Transfomation | |
|---|---|---|---|---|---|---|---|
| Infection dose (CFU.ml$^{-1}$) | Log (CFU.ml$^{-1}$) | Alive | Dead | Prop, P | Corr, P | Logit (*P*) | Probit (*P*) |
| PBS | | 14 | 2 | 0.13 | | | |
| 1x10$^4$ | 4 | 15 | 1 | 0.06 | -0.07 | | |
| 1x10$^6$ | 6 | 11 | 5 | 0.31 | 0.21 | -1.17 | 4.21 |
| 1x10$^8$ | 8 | 8 | 8 | 0.50 | 0.43 | -0.15 | 4.82 |

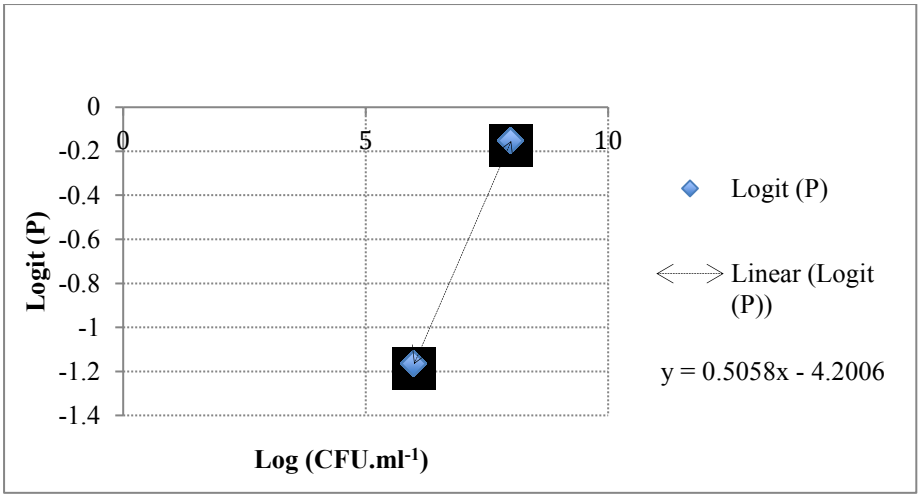



Figure S3b.1: Logit (P) plot and respective trandline equation.

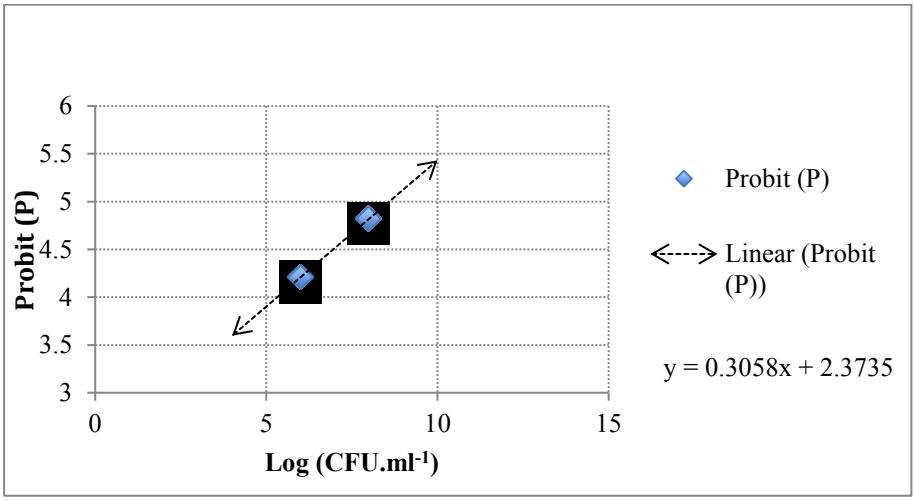



Figure S3b.2**:** Probit (P) plot and respective trandline equation.

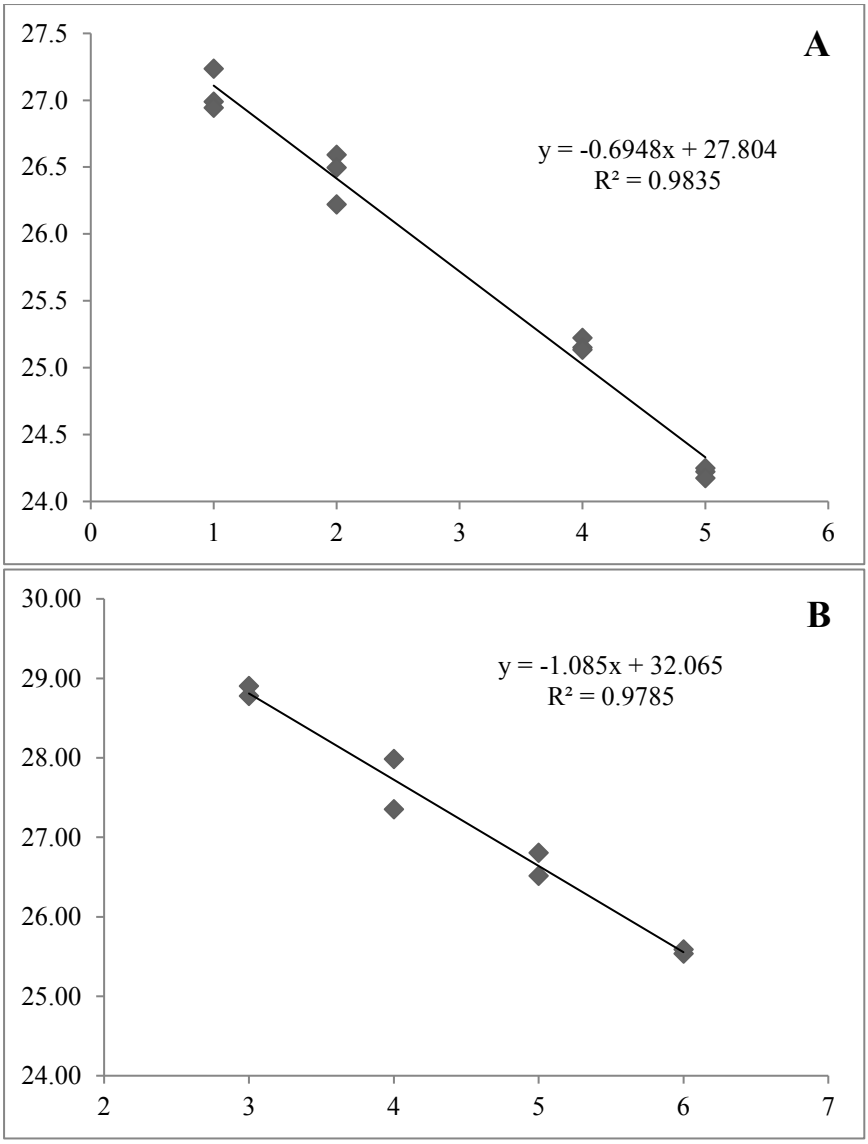



Figure S5.1: Standard curve of Log CFU.ml$^{-1}$ (horizontal axis) and threshold cycle (Ct) value (vertical axis) for specific primer for *Lactococcus* (G5) (A) and *Enterococcus* (B).

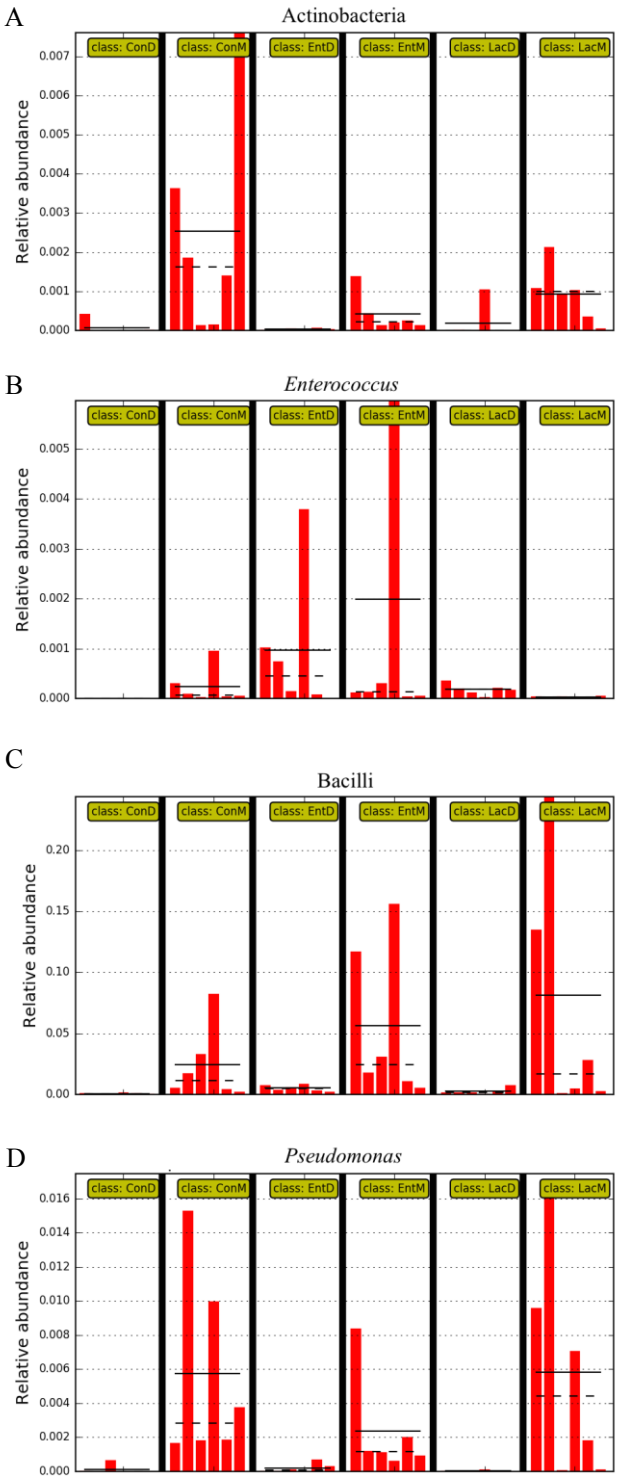



**Figure S5.2:** Differential features plots for Actinobacteria (A), *Enterococcus* sp. (B), Bacilli (c) and *Pseudomonas* sp. (D), responsible for the main difference in pirarucu (*Arapaima gigas*) gut fed with probiotic *L. lactis* subsp. *lactis* mucosa (LacM) and digesta (LacD); fed with probiotic *E. faecium* mucosa (EntM) and digesta (EntD), and pirarucu without probiotic feeding mucosa (ConM) and digesta (ConD) after 42 of probiotic feeding. Dashed line corresponds to median and full line corresponds to average between samples from the same treatment.